
University of
Southampton

University of Southampton

Faculty of Environmental and Life Sciences

School for Biological sciences

Development of an oral biofilm model to investigate the combination of mechanical forces and novel antimicrobial chewing gums on oral bacteria.

by

Katherine Emma Roe

Orcid ID - 0000-0002-6511-3087

Thesis for the degree of Doctor of Philosophy

December 2023

University of Southampton

Abstract

Faculty of Environmental and Life sciences

School for Biological sciences

Thesis for the degree of Doctor of Philosophy

Development of an oral biofilm model to investigate the combination of mechanical forces and novel antimicrobial chewing gums on oral bacteria.

By Katherine Emma Roe

Oral biofilms are the massive-multispecies communities of bacteria which live in the oral cavity. Over time there can be shifts in this community, which lead to disease; this is called dysbiosis. Oral diseases can range from gum inflammation to periodontitis and can lead to systemic diseases. People have an average of around 250 bacterial species in their oral cavity at any single timepoint; however, the total oral microbiome comprises over 700 species. With 56% of the global population experiencing an oral disease during their lifetime, the discovery of novel methods for removing oral biofilms is an important unmet need. This thesis aimed to investigate the use of antimicrobials in conjunction with chewing gum, to reduce and prevent the formation of oral biofilms.

A novel model for investigating oral biofilms, which includes aspects of the oscillatory mechanical forces experienced during chewing, was developed. A combination of literature searches and lab characterisation was used to create this novel model, building on the work completed by Wessel et al., observing bacteria being trapped in chewing gums.

Eight antimicrobial products were tested against planktonic *Streptococcus mutans*. Of these, four, cinnamaldehyde, methyl salicylate, propolis and chlorhexidine, were seen to cause a significant reduction in bacterial culturability. These were developed into chewing gum prototypes, and their antimicrobial release concentrations were characterised as ranging from 0% to 19% of the total added. *S. mutans* biofilms were then exposed to the chewing gum diffusates, but no effect was observed due to the low release concentration. However, when biofilms were exposed to chewing in the presence of cinnamaldehyde, an additive effect was detected.

Culturable counts and confocal laser scanning microscopy were used to provide insight into the effects that the antimicrobials and chewing forces have on *ex vivo* biofilms. When chewed, the culturable counts show a 3.5-log reduction in the number of culturable bacteria in both aerobic and aerotolerant species. However, a decrease was not observed when exposed to antimicrobials at 1X MIC or 4X MIC. When using confocal image analysis to investigate the changes in biofilm viability and structure, a high level of variation was observed between reactors.

Finally, this project investigated the methods of improving the DNA concentration yielded from oral biofilm extraction without the use of PCR. Fifteen extraction protocols and optimisation steps were tested. Using a phenol/chloroform extraction method with a mechanical lysis step instead of using a Qiagen PowerBiofilm kit; and swapping from a hydroxyapatite coupon to a plastic coupon were the most promising steps for improving yield.

Table of Contents

Table of Tables

Table of Figures

Research Thesis: Declaration of Authorship

Print name: Katherine Emma Roe

Title of thesis: Development of an oral biofilm model to investigate the combination of mechanical forces and novel antimicrobial chewing gums on oral bacteria.

I declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. None of this work has been published before submission

Signature: Date: 29.12.2023

Acknowledgements

My first thanks has to be to Professor Jeremy Webb and Professor Paul Stoodley, my academic supervisors without whom this project would not have been possible. I would also like to give my thanks to my industrial supervisors, Dr Beth Green, Dr Ben Dias and Dr Bob Faller, who provided industrial insights and asked thought-provoking questions during our meetings. I would like to thank BBSRC, NBIC and Mondelez for helping to fund and form this project.

I would also like to thank Professor Nick Jakubovics and Dr Halah Ahmed for providing me with a genetically modified *S. mutans* strain, which was crucial for the completion of the project. This projected required the use of the confocal microscope and Electropuls indenters, I would like to thank Dr Mark Willet and Dr Andrew Robinson for their training and continued help on these pieces of equipment.

I would like to thank my fantastic microbiology group at the University of Southampton. I have been fortunate to work alongside so many great individuals, many of whom have helped me along my PhD journey. Special thanks go to Dr Franklin Nobrega for mentoring me and keeping me on track. I wish to give my thanks to the Webb lab group, all who have helped me during my project with scientific and experimental discussions as well as the friendships formed over coffee breaks and Christmas meals.

I would also like to extend my thanks to Dr Sandra Wilks and Dr Catherine Bryant for their help and kind words throughout my project and to Professor Bill Keevil for your valuable insights during my PhD examinations. And most of all, Dr Dan Noel, who provided a vital sounding board for me throughout the past four years as well as keeping me sane, you have been and continue to be an incredible friend.

Thanks go to all the Mondelez team who have helped me throughout my PhD, thank you Beth for your continued kindness, support, and guidance. Thank you, Vicky Fuller, for all of the events and meetings you put on, helping our cohort to meet, form friendships and gain more of an insight into Mondelez. Thank you to all the team at IRIS food lab in Reading, who made my stay at Reading a valuable and enjoyable experience.

I would love to thank Ben, Dan, Rachel and Michael, during my PhD I have made an amazing set of friends who have been with me to celebrate the good times and help me through the bad times.

Without our lunchtime chats, coffee breaks, pub trips and game nights, my PhD experience would not have been as enjoyable as it was. Your friendship I will always treasure.

A special thanks goes out to my family who have helped me through this PhD, with their continued love and support, and to all my grandparents who I would have loved to see me complete my PhD. A massive thanks to my parents, Mark and Wendy, for all your trips to Southampton for meals, your willingness to listen to me talk about my project and the lifetime of help you gave me, supporting and leading me up to this point.

A final thanks goes to my fiancé Dylan who has been with me every step of the way. Thank you for providing me with such love, support, and humour during the PhD, I would not have completed this PhD without you. Thank you so much to Dylan, my family, and my friends for everything you have done for me; this thesis would have been much harder without your help.

Definitions and Abbreviations

ANOVA	Analysis of variance
ATCC	American Type Culture Collection
BHI	Brain heart infusion
CDC bioreactor	Centre for disease control bioreactor
CFU	Colony forming unit
CH_3SH	Methyl-mercaptan
CLSM	Confocal Laser Scanning Microscopy
CPC	Cetylpyridinium chloride
CSP	Competence-stimulating peptide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPSS	Diode-pumped solid-state
DLVO	Derjaguin–Landau–Verwey–Overbeek
EPA	Environmental protection agency
EPS	Extracellular polymeric substance
F. nucleatum	*Fusobacterium nucleatum*
FDA	Food and drug administration
FITC	Fluorescein isothiocyanate
GC	Guanine-cytosine
GFP	Green fluorescent protein
GM	Genetically modified
Gtf	Glucosyltransferase
HA	Hydroxyapatite
HPLC	High-performance liquid chromatography
H_2S	Hydrogen Sulphide

LAS X	Leica Application Suite X
MBC	Minimum bactericidal concentrations
MIC	Minimum inhibitory concentration
NO	Nitric Oxide
P. gingivalis	*Porphyromonas gingivalis*
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
rtPCR	Reverse transcription polymerase chain reaction
S. mutans	*Streptococcus mutans*
sBHI	Supplemented brain heart infusion
SPRE	Surface protein-releasing enzyme
SOP	Standard operating procedure
VSC	Volatile sulphur compounds
VBNC	Viable but non culturable

Chapter 1:

A literature review and introduction to oral biofilms, the current oral biofilm models and oral antimicrobials

Chapter 1 Literature review

1.1 Oral diseases and disorders

Pathophysiology of oral diseases and disorders

Oral diseases range from halitosis (bad breath) to dental caries (tooth decay). The cause of oral diseases are linked to oral microbiome dysbiosis. This is defined as a change in the ecology of the bacterial microbiome, rather than simply the presence of certain bacteria, even though specific bacteria do become more prominent in different diseases (Marsh, 2006).

Dental caries

Dental caries is a disease which includes white spot lesions and cavities and is caused by bacteria demineralizing the enamel of the tooth. This damage occurs by the bacteria producing acidic compounds as a by-product of their metabolism. This acidic environment is detrimental to the tooth enamel but not harmful to the bacteria. Therefore, the more acidogenic bacteria present, the higher the levels of acids and the faster the erosion of the enamel. This demineralisation can lead to tooth sensitivity, which is widely experienced and is an indicator of dental caries. Dental caries is associated with higher levels of oral pathogens and *Streptococcus mutans*, which is one of the primary cariogenic organisms (Aksoy, Duran and Koksal, 2006; Featherstone, 2008). This is in part due to its ability to produce acids within the oral cavity using the glycolytic pathway (Rezaei *et al.*, 2023).

Streptococcus mutans is a Gram-positive facultative anaerobic bacterium which is coccoid shaped. *S. mutans* is an early coloniser of the oral cavity, binding to the tooth's pellicle (Abranches *et al.*, 2018). *S. mutans* plays a role in dental caries because as carbohydrates are consumed, *S. mutans* metabolises them, producing acids, which demineralize the tooth's enamel, leading to tooth decay (Gross *et al.*, 2012).

Halitosis

Halitosis is the clinical term for chronic bad breath, an oral disorder which can lead to social problems. The presence of volatile sulphur compounds (VSC) is the primary cause of halitosis, with hydrogen sulphide (H_2S) and methyl-mercaptan (CH_3SH) being primarily responsible for mouth odour. Although many bacteria produce H_2S, the production of CH_3SH, especially at high levels, is primarily restricted to periodontal pathogens. Many VSCs are highly toxic to tissues even in low concentrations, so in addition to causing bad breath, they also damage the gums. CH_3SH

has also been linked to increases in the permeability of intact mucosa and also to stimulating cytokine production (Ng and Tonzetich, 1984). This cytokine production is associated with periodontal diseases such as periodontitis (Ratcliff and Johnson, 1999), thus showing halitosis as a precursor for other periodontal diseases.

Alongside VSCs, other molecules also affect oral malodour, these include volatile fatty acids and cadaverine. These molecules were discovered during the development of a halitosis measuring device that quantified VSCs only (Loesche and Kazor, 2002). All three of these groups are produced by bacterial metabolism. It was also discovered that the predominant microbiota on the tongue dorsa of healthy subjects was different from that on the tongue dorsa of subjects with halitosis, showing halitosis is either caused or affected by the bacteria present in the oral cavity (Kazor *et al.*, 2003).

Fusobacterium nucleatum has been recently found to play a major role in halitosis (Grover *et al.*, 2015; Yitzhaki *et al.*, 2018). *F. nucleatum* is a Gram-negative, fusiform rod-shaped bacterium. *F. nucleatum* is a non-motile obligate anaerobe and is highly effective in periodontal diseases due to the bacterium's ability to co-aggregate with many of the other known oral isolates (Kolenbrander, Andersen and Moore, 1989) and specifically associating with late-colonizing pathogenic bacteria including *Porphyromonas gingivalis* (Socransky *et al.*, 1998).

Gingivitis

Gingivitis' symptoms are mild gum inflammation and bleeding gums, which are caused by the build-up of plaque in and around the gingival sulcus (Page, 1986). Gingivitis is a very common oral disorder; the majority of people will experience some level of gingivitis during their life. Clinically, gingivitis can be characterised by occasional bursts of very acute inflammation. This inflammation can easily be reversed again with good dental hygiene; however, if not reversed, it can progress into periodontitis. Some of the bacteria involved in the aetiology of the disease include *Streptococcus*, *Actinomyces,* and *Fusobacterium* (Moore *et al.*, 1987; Kharitonova *et al.*, 2021), however it has also been noted that with gingivitis progression there is an increase to microbial diversity (Al-Kamel *et al.*, 2019).

Periodontitis

Periodontitis is a more severe infection of the gums than gingivitis, and is irreversible. Initial symptoms include inflamed gums, which progresses to receding gums and then to degradation of the gums and alveolar bone, which support the teeth.

Periodontitis is also thought to be caused by oral dysbiosis, where there is an overall change from Gram-positive aerobic bacteria to Gram-negative anaerobic bacteria (Marsh, 1994). There is often

an increase in *P. gingivalis* observed. *P. gingivalis* produces a myriad of virulence factors (Hajishengallis, Darveau and Curtis, 2012), leading to the destruction of periodontal tissue and alveolar bone (How, Song and Chan, 2016). The inflammation caused by tissue destruction is also a selection factor for certain bacteria, which in turn increases inflammation, increasing their dominance in a positive feedback loop, similar to what is seen in dental caries (Bartold and Van Dyke, 2019).

P. gingivalis is a key pathogen associated with periodontitis; it is a Gram-negative rod-shaped bacterium that is a non-motile, obligate anaerobe. *P. gingivalis* is usually present in high numbers during disease, and as an anaerobe, it would be present deep in the oral biofilms (Shaowen Zheng *et al.*, 2021). This speaks to the importance of frequent biofilm management.

Candidiasis

Candidiasis, commonly known as oral thrush, is caused by the increased presence of C*andida spp.* (Gozalbo *et al.*, 2004)*;* this is an opportunistic infection*.* The symptoms are a red oral cavity with white patches, which can progress to lesions. The risk factors for candidiasis included impaired salivary gland function, a high carbohydrate diet and immunosuppressive conditions (Millsop and Fazel, 2016). The C*andida* genus contains around 200 different species (Odds, 1987), many of which are commensal within the gut, oral cavity and skin (Hube, 2004).

1.2 Systemic diseases

There is a strong link between poor oral health, especially gingivitis and periodontitis, and a range of systemic diseases (Kim and Amar, 2006; Botelho *et al.*, 2022). These systemic diseases are usually due to the chronic inflammation caused by oral diseases. There are two main ways systemic diseases are caused by oral bacteria, firstly, they can enter the bloodstream and cause damage, or secondly, as the inflammatory response tries to fight the periodontal infection, it attacks the wrong area causing new issues (Li *et al.*, 2000). This is considered a complex multiphase disease due to all the possible follow-on diseases caused by periodontal infection. Whilst there is some positive links between treating oral diseases to reduce systemic diseases, it ranges significantly between each disease, with some papers contradicting each other (Offenbacher *et al.*, 2009; Merchant *et al.*, 2018; Beck *et al.*, 2019).

Alzheimer's disease

Recently the link between periodontitis and Alzheimer's disease has been increasingly explored. One group used a mouse model with *P. gingivalis* and its' associated toxins called gingipains (Dominy *et al.*, 2019). These gingipains can degrade human proteins, leading to misfolded

proteins, which are thought to be involved in Alzheimer's disease. Mice treated with these gingipains were seen to develop degenerate brain cells, however, when pre-treated with a gingipains neutralising agent, the mice maintained healthy brain cells. This shows that *P. gingivalis* might a precursor of Alzheimer's disease, and therefore, the high levels present in periodontitis may be a contributing factor. If this is one of the causes of Alzheimer's disease, this would open a completely new set of possible preventative treatments aimed at removing the bacteria or neutralising the gingipains (Dominy *et al.*, 2019).

Diabetes

People with diabetes face a higher prevalence and severity of gingivitis and periodontitis. This is because they have a weaker immune system and thicker blood vessels, reducing protection against oral disease and the flow of nutrients. Diabetes also causes a higher concentration of oral glucose which provides the bacteria with higher nutrient levels, which, in turn, increases the risk of oral dysbiosis and disease (Mascarenhas, Fatela and Barahona, 2014).

Cardiovascular disease

In many studies, it has been suggested that periodontal disease is linked to various types of heart disease, including heart failure and atrial fibrillation (Dhadse, Gattani and Mishra, 2010; Chen *et al.*, 2016). Chronic periodontitis is considered a cardiovascular risk factor. It is thought that periodontal inflammation triggers systemic inflammation. This is combined with the bacteria entering the blood circulation, promoting atheroma plaque development and progression (Lee *et al.*, 2019).

Low birth weight

Low birth weight is a major problem which occurs globally and has been linked to oral disease. Low birth weight is defined as weighing less than 2,500 g when born. As with the other systemic diseases, the first step is the bacteria invading the bloodstream and causing systemic inflammation. This inflammation can target inappropriate sites around the body, including the foetus of pregnant women. This can lead to both premature labour and low birth weight, both of which can have serious consequences on the baby (Saini, Saini and Saini, 2010). In addition to systemic inflammation, bacteria in the blood stream can be transported to the foetus, which then in turn leads to a systemic response against the bacteria in the foetal-placental area (Figuero, Han and Furuichi, 2020). Being born prematurely or at a low birthweight can then lead to breathing problems, neurological problems, an inability to maintain temperature or gain weight, and sudden infant death syndrome (Goldenberg and Culhane, 2007).

Aspergillosis

Aspergillosis is an opportunistic infection caused by the inhalation of aspergillus mould (*Aspergillus spp*.) (Brakhage, 2005). This disease rarely occurs in healthy people, usually infecting immunosuppressed people, people who have tuberculosis or have a lung condition such as asthma. It causes a wheezing cough, shortness of breath and weight loss (Shibuya *et al.*, 2004). There are hundreds of *Aspergillus* species which are spore-forming moulds (Paulussen *et al.*, 2017). *Aspergillus spp.* can be harmful and infectious (Iversen *et al.*, 2007) or beneficial and used in fermentation (Papagianni, 2007).

With all these diseases in both the oral cavity and systemically, the importance of good oral hygiene and improving oral practice is ever more apparent. According to the global burden of disease database in 2019 just over 56% of people globally experienced an oral disease (Abbafati *et al.*, 2020). This equates to 4.48 billion people suffering from preventable and treatable diseases. Oral diseases are often untreated in low and middle-income countries due to the cost of treatment, and as oral diseases are due to microbiome dysbiosis, leaving them untreated will only lead to disease progression and more extreme oral diseases, with increasing oral problems and pain (Peres *et al.*, 2019). Finding cheaper, easily accessed oral treatments are essential for improving oral health globally and reducing the systemic diseases associated with poor oral health.

1.3 Microbiology of the oral diseases

Healthy mouths are colonised with up to 300 different species of bacteria

A microbiome is a community of microorganisms, including bacteria, fungi, and viruses, that inhabit a specific environment (Shanahan, Ghosh and O'Toole, 2021). Microbiomes can be any size, from the whole human microbiome (all microorganisms in and on the human body) to a single organ, such as the oral microbiome (all microorganisms in the oral cavity). The human oral cavity comprises seven different zones: the teeth, gingival sulcus (gums), cheeks, tongue, tonsils, and soft and hard palates (Dewhirst *et al.*, 2010).

The human mouth is teeming with bacteria, with around 270 species of bacteria present in a healthy individual's mouth at any time (Zaura *et al.*, 2009), making it the second-largest organ microbiome after the gastrointestinal tract (Verma, Garg and Dubey, 2018). These bacteria can be as specific to an individual as a fingerprint (Edlund *et al.*, 2017). In total, over 700 bacterial species have been discovered in the human oral cavity. Many of these different species have been found through the use of 16S sequencing (Aas *et al.*, 2005).

Sequencing the oral microbiome

In 2018, it was estimated that 70% of the bacteria in the oral cavity were culturable (Verma, Garg and Dubey, 2018), leaving 30% unculturable and needing sequencing to characterise the remaining oral microbiome. 16S sequencing has been regularly used to discover which bacteria are present in the oral cavity. This is due to the fact that all bacterial species have the 16S rRNA, but it varies enough between species to allow for species identification. 16S sequencing can be run on as little as 30 ng of total deoxyribonucleic acid (DNA) with risk, however there is a recommended quantity of 200 ng (Novogene, personal communication, 23rd). This makes 16S sequencing a handy tool for microbiome characterisation and identifying which species are present. In addition to 16S sequencing, 18S sequencing has been carried out in parallel to explore the presence of eukaryotic species present in the oral cavity (Baehren *et al.*, 2023). For the identification of fungal species; Internal Transcribed Spacer (ITS) sequencing could be carried out (Ghannoum *et al.*, 2010), this would provide sequence specificity.

Polymerase chain reaction (PCR) has been used in conjunction with 16S sequencing to increase the DNA present for sequencing (Kazor *et al.*, 2003; Ahn *et al.*, 2011). This is beneficial when investigating the oral cavity to ensure the bacteria present in lower quantities can still be sequenced. However, using PCR to amplify DNA does come with its pitfalls, including GC bias and the construction of artefacts (Acinas *et al.*, 2005; Laursen, Dalgaard and Bahl, 2017).

A secondary sequencing method is whole genome, shotgun, sequencing, which requires more extracted DNA for sequencing, from 100 to 1000 ng, to achieve a good read depth. Whilst this method elucidates which species are present; it provides a much deeper level of information. As you have the whole genome of the cells, it provides information on any variations of the genome, and this can give insight into how treatments or external stresses are affecting the cells. This has been used to add genomes to the human oral microbiome database to help distinguish healthy oral microbiomes (Caselli *et al.*, 2020).

Reverse transcription polymerase chain reaction (rtPCR) can be used to determine if species are present using transcribed mRNA. It uses primers of genes specific to a species, then amplifies the DNA present, with the capability of amplifying the DNA from a single cell (Devienne *et al.*, 2018). This has previously been used to identify bacteria in the oral microbiome (Jung *et al.*, 2018).

Core oral microbiome

Using 16S rRNA sequencing, it was found that *Streptococcus* species are one of the most abundant genera present in the healthy microbiome (Bik *et al.*, 2010). However, one-third of oral bacteria are as yet un-cultured, and without being able to culture them, we have to make

assumptions based on their genetics and assumptions about their prevalence in diseases (Vartoukian *et al.*, 2016). Metagenomic sequencing provides a more in-depth information on the bacteria present, as you receive the full genome of the bacteria rather than just if the species is present (Lazarevic *et al.*, 2009).

Within the 70% of the bacteria in the oral cavity which were culturable in 2018, 57% of those were assigned into six broad phyla groups *Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria,* and *Spirochaetes*. These bacteria are known to make up 96% of the total oral bacteria present in a healthy mouth (Verma, Garg and Dubey, 2018). The other four percent are in much smaller quantities however, they provide 30% of the diversity of bacteria. These percentages are for the total oral microbiome, however each of the seven zones will have its own slightly different microbiome (Aas *et al.*, 2005). The predominant bacterial genera in the oral cavity were *Streptococcus, Gemella, Abiotrophia, Granulicatella, Rothia, Neisseria, Prevotella*, and *Proteobacteria* (Zaura *et al.*, 2009; Bik *et al.*, 2010).

Figure 1 (Kolenbrander *et al.*, 2010) shows the biogeographical species distribution. It shows that *Streptococcus* species are the main initial colonisers of the dental pellicle. The secondary colonisers are more varied, with some co-aggregating with many species. The secondary colonisers are called bridging species as they are capable of binding to the early and late

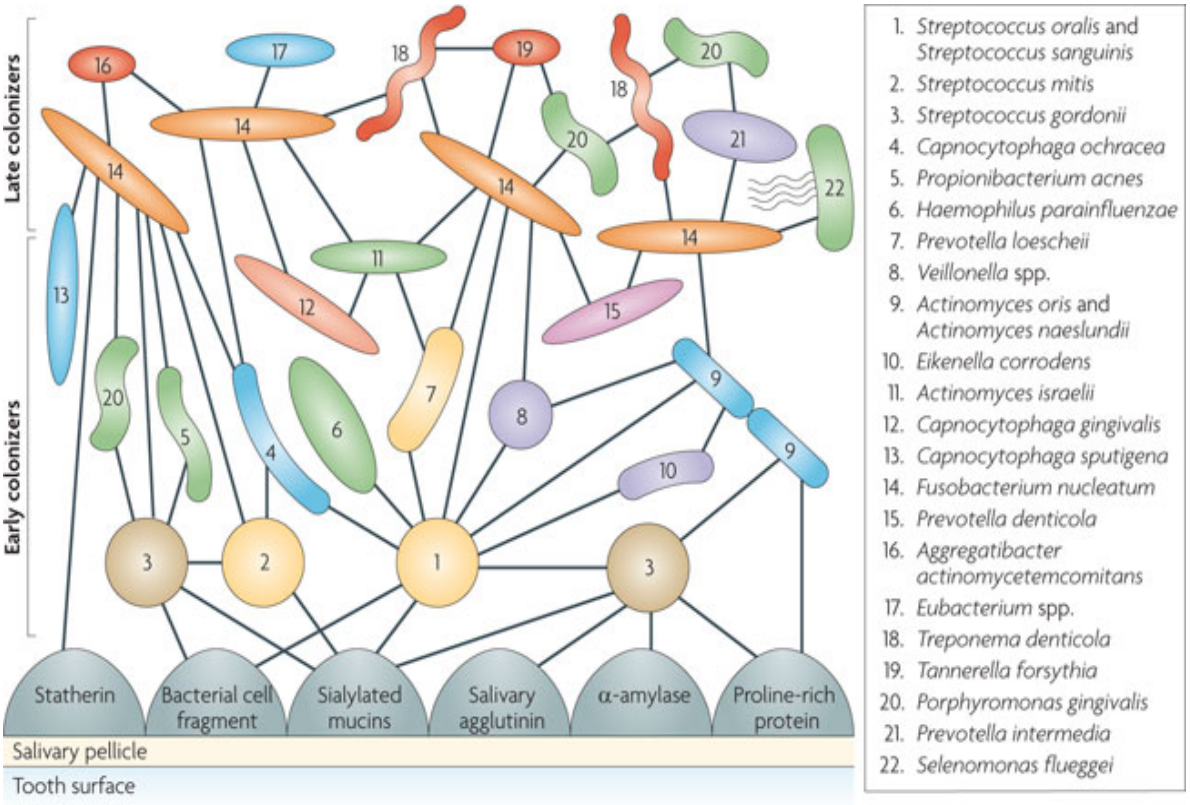


Figure 1: Shows the spatiotemporal positioning of key bacterial species in relation to the tooths surface (Kolenbrander et al., 2010). Image reproduced with the permission of Springer Nature.

colonisers. One example of a bridging species that can co-aggregate well is *F. nucleatum*, shown in Figure 1 as number 14, it can co-aggregate with many other bacterial species. This ability to co-aggregate is what makes these bridge species so effective. Only 22 species are shown in this diagram, but with up to 300 in the mouth, *in vivo* the situation is even more complex.

The dental pellicle is key for biofilm formation, this is what the early colonisers attach to. It only takes a few minutes for the pellicle to adsorb onto the teeth and it is continually remodelling (Hannig, 1999). The pellicle is formed from proteins, glycoproteins, enzymes, and mucins (U. Lendenmann', J. Grogan1, 2000). Glucosyltransferases (Gtf) are a group of enzymes commonly found in saliva and the pellicle. They are highly produced by *Streptococcal* species. Due to their binding capabilities, they are hypothesised to play a key role in bacterial attachment (Bowen and Koo, 2011).

Dysbiosis causes diseases

Microbial dysbiosis occurs when there is a decrease in bacteria that are beneficial to good oral health and an increase in pathogenic bacteria (Sudhakara *et al.*, 2018). This tends to be a slow process that occurs over time, meaning it can be reversed by good dental hygiene. However, prolonged bad oral hygiene will lead to complete dysbiosis and a highly pathogenic oral microbiome (Nath and Raveendran, 2013).

When in dysbiosis, certain disease-associated bacteria become more predominant, with common networks of co-occurring bacteria. These networks can be assigned to the 'orange' and 'red' complexes. The orange complex is the first stage of dysbiosis, with a positive feedback loop that encourages the progression of disease. This progression leads to the red complex stage and its associated bacteria thriving. The orange complex contains bridging species and includes *F. nucleatum*, *Prevotella spp.,* and several *Campylobacter spp.,* among others. The red complex consists of; Tannerella forsythia, *Treponema denticola,* and *P. gingivalis* (Socransky *et al.*, 1998). The bacteria in the red complex are species highly associated with oral disease (Thurnheer, Belibasakis and Bostanci, 2014).

Microbial dysbiosis is the community shift from a healthy microbiome to a more pathogenic microbiome. There are several factors that can help shift the microbiome towards this state of dysbiosis, one of which is the microbiome environment. The growth of thick biofilms leads to the generation of anaerobic pockets deep in the biofilm. Pathogenic oral bacteria tend to thrive in anaerobic environments leading to dysbiosis and disease (Benachinmardi *et al.*, 2015). Some of these bacteria excrete acidic compounds, others deaminate proteins increasing pH creating an

environment which is conducive to the inclusion of more pathogenic bacteria into the biofilm and the further erosion of teeth and gums.

Another factor is the human diet, the oral biofilm differs from other biofilm environments due to the frequent sharp peaks in oral glucose, sucrose and other sugars, alongside other nutrients after eating. Sucrose especially has been liked to cariogenic progression, more so than glucose (Leme *et al.*, 2006; Du *et al.*, 2020). This causes the dental biofilms to grow quickly and be thicker, making them harder to control and remove. It has been shown that the intake of sugars, such as glucose and sucrose, causes a fall in pH and a long-term change toward more acidogenic bacteria in carious plaque (Marsh, 1994; Leme *et al.*, 2006).

1.4 Biofilm growth in the oral cavity

Overview of the biofilm life cycle

A simple definition of a biofilm is “communities of microorganisms attached to an interface” (O’Toole, Kaplan and Kolter, 2000). However, there are many discussions on the finer details of biofilms, with some extending the definition to include the production of a matrix (Kannan *et al.*, 2017). Other definitions also include aggregates and bacteria attached to bacteria as a biofilm stage (Sauer *et al.*, 2022). In most biofilm *in vitro* studies, only bacteria are included in making up the biofilm; however, *in vivo* biofilms can include fungi and protozoa. These microorganisms, which are not included in most studies, may alter biofilm structure, adherence or even the biofilms’ ability to be removed. Stefanakis showed that the presence of protists increases biofilm removal efficiency (Stefanakis *et al.*, 2014).

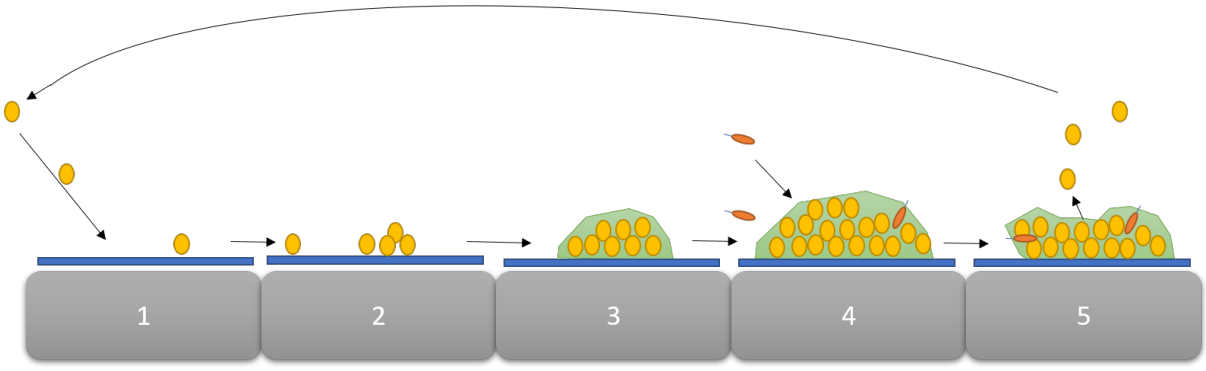


Figure 2: Biofilm life cycle, showing the five main stages of biofilm formation in the oral cavity with the salivary pellicle present. Stage one has the bacteria attaching to the salivary pellicle on the tooth. The early stages of biofilm development occur, creating a monolayer of bacteria (stage 2). This monolayer then binds irreversibly, this then matures, and the production of extracellular polymeric substance begins protecting the biofilm (stage 3). The biofilm thickens and encourages binding with other species, as shown in stage four. Finally, in stage five, bacteria start to disperse, allowing the inner biofilm access to nutrients and the dispersed bacteria to begin the cycle again.

Attachment

Attachment is the first phase in the biofilm life cycle and is shown in Figure 2 stage 1. Planktonic bacteria move towards the surface and start initial attachment, at this point, it is reversible. When the bacteria are 5-20 nm away from the surface, electrostatic and hydrophobic charges interact, holding the bacteria in place. Once within 0.2-2nm from the surface, the bacteria use specific binding such as polymer bridging, chemical binding (non-specific), or pili with adhesions to attach to the surface.

The Derjaguin–Landau–Verwey–Overbeek (DLVO) theory involves Van der Waal forces and the electric double-layer theory to explain particle coagulation and stabilisation (Hermansson, 1999). At a distance, repulsive forces are more dominant than attractive forces, however, as the distance between the particles decreases the attraction becomes more dominant over the repulsion, pulling the particles together. This can be used to explain bacterial attachment, where one particle is a bacterium and the other is either a bacterium or a surface (Bos, van der Mei and Busscher, 1999).

Pili are present in both Gram-negative and Gram-positive bacteria, and they can help mediate attachment to surfaces, other bacteria, and host cells (Sauer *et al.*, 2000) with a high level of specificity (Klemm and Schembri, 2000). There are five main classes of pili in Gram-negative bacteria and two classes in Gram-positive bacteria, distinguished by their expression and assembly(Telford *et al.*, 2006; Hospenthal, Costa and Waksman, 2017). Pili are also important in oral biofilms with some key pathogens, including *Aggregatibacter actinomycetemcomitans* and *P. gingivalis*, using them to aid attachment (Wu and Fives-Taylor, 2001).

In the oral cavity, a salivary pellicle coats the teeth immediately after brushing, it contains albumin, glycol-proteins, and mucins (Hannig and Joiner, 2006). Often the components of the salivary pellicle come from microorganisms in the oral cavity (Siqueira, Custodio and McDonald, 2012). These proteins act as substrates for which bacteria with high-affinity adhesins can bind. These are important interactions for the early colonisers such as *Streptococcal* species (Bowen and Koo, 2011). Some *streptococcal* species, such as *S. mitis,* also require the presence of salivary amylase for binding to, to colonise mammalian oral surfaces (Scannapieco, Solomon and Wadenya, 1994).

Early development

Once irreversibly adhered, these cells start to divide creating expanding cell clusters. At this point, the formation of an extracellular polymeric substance (EPS) occurs, made up of many components including proteoglycans, fibrous proteins, and extracellular DNA (Wingender, Neu and Flemming,

1999). These components play numerous roles in biofilm structure and function, holding the cells together and protecting the cells from shear stress and harmful molecules, both from the host and from antimicrobials. Figure 2 stage 3 shows how the EPS also provides a scaffold for the biofilm to grow on as well as containing nutrients for the bacteria to utilise.

Mature biofilms

As the EPS is produced, it begins to form a matrix around the bacterial cells allowing the bacteria to form micro-colonies, which are defined as clusters of bacteria surrounded by EPS, and collectively they make up the biofilms as seen in Figure 2 stage 4. Biofilm architecture is species and environment-dependent, with some species exhibiting large mushroom-shaped microcolonies and others forming small towers. However, there is an ongoing debate as to whether these structures are artefacts of the *in vitro* culture conditions, as they change depending on media choice (Tolker-Nielsen, 2015).

The presence of EPS also allows for bacterial communication by quorum sensing, the transfer of genetic material using horizontal gene transfer, as well as maintaining useful nutrients and enzymes. Quorum sensing is the communication between cells using the presence and recognition of signalling molecules and their concentrations. In the early coloniser, *S. mutans,* quorum sensing uses a competence-stimulating peptide (CSP) and its partner ComD/ComE signal transduction system (Li *et al.*, 2001). This signalling pathway has been shown to affect cell viability and is important for biofilm formation (Yung-Hua, 2002; Zhang *et al.*, 2009).

Horizontal gene transfer is the movement of genetic material from one organism to another, in this case, bacteria. It can occur via three mechanisms, the first being transformation when it is taken up from the environment. The second is via direct transfer from another cell, conjugation, with the third being transduction, where bacteriophages transfer it from one cell to another (Von Wintersdorff *et al.*, 2016). This is a common method for how antibiotic resistance is spread between bacteria, and it occurs at an increased rate in biofilms. The increased rate is due to the prolonged proximity of the bacteria (Hendrickx, Hausner and Wuertz, 2003).

Mature biofilms have several different protection mechanisms; the EPS not only holds the cells together and to the surface but also helps to protect the cells from antimicrobial compounds. It reduces the perfusion of drugs through the biofilm, so whilst the cells on the outer layer of the biofilm may not survive, the rest of the biofilm is exposed to a much-reduced concentration, allowing for bacterial survival. Biofilms have other protective properties, such as the dormancy of some bacteria which can be caused by stress. These have a reduced metabolism which means

many of the drugs that rely on bacteria metabolising them are ineffective (Wood, Knabel and Kwan, 2013).

Biofilms usually contain channels, which flow around the microcolonies allowing water and nutrients to flow around the biofilm. These channels allow the centre of the biofilms to still get access to higher levels of nutrients, in addition to perfusion through the biofilm, allowing the non-dormant bacteria to survive. Channels can be seen in Figure 3 (Stoodley, Debeer and Lewandowski, 1994), showing the flow of a bead through a channel in a biofilm.

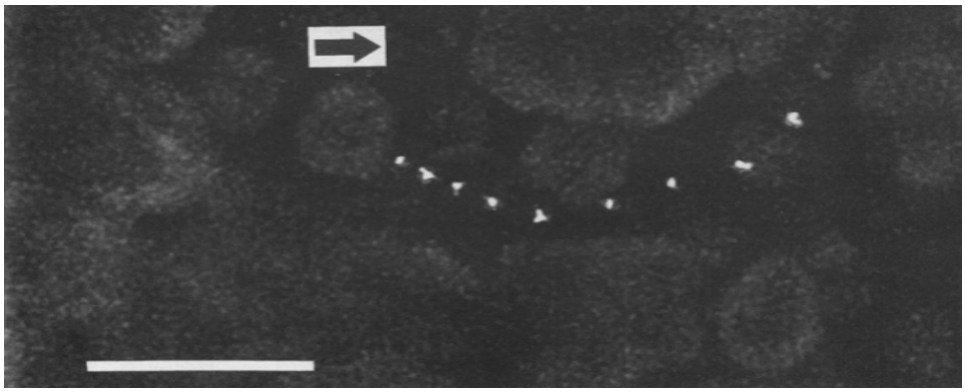


Figure 3: Superimposed time series image showing a single bead moving through the channel of a biofilm. Bulk flow is indicated by the arrow and the scale bar = 100 μM. This emphasizes the presence of channels in the biofilm and how nutrients and water can flow through the biofilm. This image is reproduced with permission from (Stoodley, Debeer and Lewandowski, 1994).

Late-stage development

Biofilms keep growing until their structures have grown so large that nutrients, available space to grow into, or mechanical forces removing the biofilm have become limiting factors. Biofilms will then start to produce molecules that will kill a small part of the biofilm, which leads to bacterial dispersal, as shown in Figure 2 stage 5. For some bacteria, this molecule is nitric oxide, and studies have shown that when NO is produced, oral biofilms have increased bacterial counts (Barraud *et al.*, 2014), this may be due to increase bacterial activity, waking up the dormant cells before dispersal. This allows the bacteria deep in the biofilm to be able to access nutrients, as well as start the biofilm life cycle again as the dispersed bacteria will move on and potentially reattach elsewhere.

There are three ways biofilms undergo dispersal; erosion, sloughing, and seeding. Erosion and sloughing can be passive or active. Erosion is the slow release of bacterial cells as the biofilm grows, whereas sloughing depicts the sudden release of a large number of cells. Sloughing usually occurs during the later stages of biofilm growth (Lappin-Scott and Bass, 2001). Seeding dispersal refers to the release of a large number of single cells or cell clusters from the centre of the biofilm's microcolony (Ma *et al.*, 2009).

For *S. mutans* dispersal, one key molecule that plays an important role is the proteinase, surface protein-releasing enzyme (SPRE), which causes dispersal by releasing proteins from the cell's surface. SPRE also degrades salivary receptor P1, which is a key attachment protein; this degradation aids *S. mutans* dispersal (Vats and Lee, 2000).

Forces on biofilms

Biofilms are subject to mechanical forces such as shear force, where shear force is the force asserted on the biofilm from the surroundings. Shear force affects the biofilms in the form of flow force from liquid running over the biofilm at a solid-liquid interface. This can cause alterations to shape and structure as well as potentially dislodging the biofilm from the surface (De La Fuente *et al.*, 2007). Biofilms may alter their characteristics depending on the environmental niche they are in. If there is a high drag force due to high flow or pressure, the bacteria will have to exert more energy resisting the flow (Bakker *et al.*, 2004).

Shear forces have large effects on the structure of the EPS; however, they also have effects on nutrient channels in the biofilm. They can create nutrient channels by causing the biofilm to buckle, creating a channel through or under the biofilm. This can be beneficial to the biofilms by allowing better access to nutrients in the depth of the biofilm (Wilking *et al.*, 2013). This occurs by increased diffusion into the biofilms via these tunnels.

Bacteria can also form streamers, where a microcolony is attached to a surface at one end, and the rest moves in the flow. They can occur in turbulent waters and laminar flow around corners in pipes and nature (Rusconi *et al.*, 2010). These streamers are present in ecosystem processes and biofouling. They have also been seen to occur in mixed bacterial biofilms. They also can occur in medical devices; the EPS tails floating in the fluid can catch cells and other debris leading to blockages and medical complications (Drescher *et al.*, 2014).

Mechanical forces can also have a large effect on molecular movement, which could, in turn, affect quorum sensing. Usually, cells sense local autoinducer molecule concentrations and can then assess their density and react appropriately. However, if there is a heavy flow over the biofilm and through biofilm channels, the concentration may be reduced, prohibiting them from quorum sensing (Kirisits *et al.*, 2007). It is thought that microbes may use this to their advantage to detect flow rate, which may, in turn, provide information about the growth potential of the area (Cornforth *et al.*, 2014). Flow may be highly beneficial to biofilms in the dispersal phase; the shear stress may be able to promote dispersal by adding force to weakened areas, and the flow will then be able to carry the dispersed biofilm to a new area for colonisation (Kaplan, 2010).

The mechanical force of chewing, especially chewing gum, causes biofilm removal. This is due to the stress exerted on the biofilm by the gum; sections of biofilms also become trapped in the gum and can be pulled off (Wessel *et al.*, 2015). The mechanical force of chewing, however, will also affect the architecture of the remaining biofilm on the tooth's surface and in the ridges and interproximal space. Along with increased oxygen, the biofilms will be exposed to higher levels of nutrients, which may aid biofilm growth in the harder-to-clean spaces.

Depending on the environmental niche, the bacteria will create a different type of biofilm. This is seen in oral biofilms, as there are seven different areas of the oral cavity which have different flow levels. Fernandez's experiments show that, depending on which area the bacteria are sourced from, their growth rates and architectures differ, and these are also affected by the shear rate. The different oral bacterial communities have optimised their biofilm growth for different shear flows (Fernández *et al.*, 2017).

Viable but non culturable

Another state bacteria can enter, separate from biofilms, is viable but non-culturable (VBNC). This is where the cells are still metabolically active, at a reduced level, with intact membranes and control of their gene expression but are un-culturable and cannot reproduce (Del Mar Lleo *et al.*, 2000; Oliver, 2000). This dormant-like state was first investigated in 1984 (Roszak, Grimes and Colwell, 1984) and affords the bacteria an increased survival rate (Ramamurthy *et al.*, 2014). Entry into the VBNC state is thought to be due to exposure to stress (Besnard *et al.*, 2002; Pasquaroli *et al.*, 2013), as many of the genetic and phenotypic changes that occur are to reduce the effects of stress. These changes include decreased cell size for starvation (Watson, Clements and Foster, 1998) and enhanced efflux systems for dealing with exposure to antimicrobials such as chlorine (Ye *et al.*, 2020).

1.5 Oral treatments

Many current treatments use a combination of mechanical forces and antimicrobial activity. This combination allows the antimicrobial agents access to deeper parts of the biofilm where more pathogenic bacteria are present.

Tooth brushing

Using a toothbrush removes bacteria from the hard and soft surfaces of the mouth. However, bacteria will start to build up again quickly. Brushing your teeth is the main oral hygiene advice given by dentists; brushing twice daily for two minutes allows for the mechanical removal of any

built-up biofilms as well as the delivery of key ingredients such as fluoride to reduce biofilm formation and aid remineralisation (NHS, 2018).

Many brands of toothpaste include fluoride as a additive. Fluoride has a less well-known mechanism of action in oral biofilms; it has been shown to reduce demineralisation by replacing the hydroxyl group in hydroxyapatite, making it less soluble at a low pH. However, fluoride also has antimicrobial activity, although the mechanism for this is not fully understood. This tooth protection may be caused by reduced acid production, EPS volume or a change in the balance between re/demineralisation (Thurnheer and Belibasakis, 2018). Additionally, alongside fluoride, many kinds of toothpaste include detergents, for example, sodium lauryl sulphate, which helps the water and oil mix, preventing oral bacteria from surviving on the toothbrush until the next brushing (Quirynen *et al.*, 2008).

The quantity of bacteria removed from the oral cavity whilst brushing is difficult to measure, one paper suggests that 10^8 bacteria are removed (Quirynen *et al.*, 2001). This number was calculated from the bacteria present on a toothbrush after brushing without toothpaste (Quirynen *et al.*, 2003). The problem with this assumption is that the bacteria on the brush does not account for any dislodged oral biofilm that does not get attached to the toothbrush and may be swallowed or spat out. The secondary problem is that the bacterial colony forming unit (CFU) count was calculated from the bacteria from the brush being plated on brain heart infusion (BHI) and stored in both anaerobic and aerobic incubators. However, many bacteria do not grow on BHI, and this is true for many pathogenic oral species, and this also does not account for any unculturable species. The removed oral biofilms may also be removed in clumps leading to a reduced culturable count.

Due to the challenges in measuring bacterial removal, the number of bacteria removed is likely to be much higher than 10^8 and also have a significant margin for error. Especially as a simple CFU count before and after treatment/brushing is not feasible. To overcome the problem of culturability, 16S RNA sequencing can be used to identify which species are present alongside quantitative polymerase chain reaction (qPCR) to determine the quantity of the species. However, full sample collection from the oral cavity is still very hard to achieve due to the hard-to-reach areas of the oral cavity, and each of the seven areas have different microbiomes.

Mouthwash

Mouthwashes are incorporated into daily oral hygiene routines to improve oral health. However, studies show that mouthwashes vary hugely in efficacy, depending on which antimicrobial compounds they include (Mat Ludin and Md Radzi, 2001). Some studies show that antimicrobial

compounds such as cetylpyridinium chloride (CPC) can help to reduce the culturability of oral biofilms in *in vitro* studies. When CPC was used to pre-treat hydroxyapatite discs, they showed significant inhibition of biofilm formation (Latimer *et al.*, 2015; Mao *et al.*, 2020). Some mouthwashes also contain fluoride, which will help to prevent demineralization and re-mineralise tooth enamel.

Flossing/Interdentals

Flossing works by the mechanical removal of biofilms from between the teeth in areas that can be hard to reach using a toothbrush. This makes it a good addition to current oral hygiene techniques. As well as floss, interdental brushes work similarly, and both have been shown to reduce the risk of gingivitis, with interdental brushes having a slightly more beneficial effect than floss (Worthington *et al.*, 2019).

There has also been a more recent development of using water jets to floss, an example being the WaterPik. This is 18% more effective at plaque removal than regular interdental brushes when used in conjunction with teeth brushing (D Lyle , 2016).

Experimental vaccines

A bivalent vaccine against *F. nucleatum* and *P. gingivalis* has been produced and shown to be able to induce a protective immune response, which inhibits alveolar bone loss caused by oral dysbiosis in a mouse model (Puth *et al.*, 2019). This is a very promising improvement in oral hygiene; however, in the long term, this may lead to a change in which species are present in the oral cavity and cause alternative diseases.

Chewing gum

Chewing gum is thought to remove bacteria by the mechanical action of the gum rubbing against the tooth. Chewing gum has been observed to both remove plaque and reduce new plaque formation in the upper palatal and lower buccal tooth areas (Takahashi *et al.*, 2003). Furthermore, chewing gum has been observed to trap up to 10^8 bacterial cells (Wessel *et al.*, 2015), which is a similar efficacy to current estimates from the use of a new toothbrush with no toothpaste. However, the total amount of bacteria dislodged from teeth could be higher because of the increased salivary production rate caused by chewing gum. This means that any dislodged plaque is more likely to be either trapped in the gum or removed and not have a chance to reattach.

Chewing gum is often used as a reference food in experiments investigating chewing rate and force. Its use in these experiments is due to its elastic properties and the fact it does not break down over time (van der Bilt and Abbink, 2017). A great deal is known about the chewing rate and the force involved in chewing (Kohyama *et al.*, 2004; Steiner *et al.*, 2009).

However, much less is known about the physical interface between chewing gum with the surface of the tooth, on which the biofilms form. Connections can be made between the shear force of the chewing gum and saliva on the biofilm and biofilm elasticity acting to protect the biofilm (Stewart, 2014). A literature search was completed on Google Scholar and Pubmed using the key terms “oral biofilms”, “chewing gum”, “interaction”, “forces”, “teeth”, “plaque”, and “oral cavity” in different combinations. Upon reading the titles and abstracts, no appropriate papers were found to discuss the direct interaction between chewing gum, the biofilm and teeth as when all combined there may be variations to the effects.

There is currently a lack of information regarding chewing gum additives and their impact on the oral microbiome. There are six common additives used in chewing gum: xylitol, sorbitol, maltitol, mannitol, acesulfame potassium, and aspartame (Drugs, 2019). However, only xylitol, sorbitol, and maltitol have any information on their effects on oral biofilms. There has been extensive research on xylitol; however, the proposed effects of xylitol on oral biofilms are often contested; the papers tend to disagree on the degree of the effect or even if it causes one at all (Söderling *et al.*, 2011; Nayak, Nayak and Khandelwal, 2014; Rafeek *et al.*, 2019). There are observations that xylitol aids remineralisation by increasing the salivary rate and inhibiting bacteria growth (Nayak, Nayak and Khandelwal, 2014). Furthermore, patients with high cariogenic risk treated with xylitol displayed “improvement in salivary parameters and a decrease in bacterial biofilm activity” (Pancu *et al.*, 2017).

Some studies show that chewing gum that contains xylitol three times a day can lead to a statistically significant reduction in the quantity of *S. mutans* (Çaglar *et al.*, 2007; Söderling *et al.*, 2011). However, other groups contend that the exposure of *S. mutans* to xylitol caused no inhibition of viability (Decker *et al.*, 2014). These variations may be due to the use of different xylitol concentrations, length of exposure or volunteer compliance. More current literature indicates that xylitol does have an effect (Wu *et al.*, 2022), based on this and the in-depth gene effect conclusions of earlier studies, it is highly likely that xylitol is effective on both *S. mutans* and oral biofilms.

Maltitol, another additive, has been shown to lower the abundance of several bacteria key in early biofilm formation, whilst the gum base control only marginally changed plaque microbiota composition (Keijser *et al.*, 2018). Sorbitol does not significantly change bacterial plaque composition, but it did affect several streptococcal species. Sorbitol was seen to increase the abundance of *Streptococcus cristatus,* an oral bacterium known to inhibit bacterial growth for bacteria associated with chronic periodontitis. This could be beneficial to the oral microbiome,

especially as sorbitol does not increase the abundance of *S. mutans,* an oral pathogenic bacterium (Rafeek *et al.*, 2019).

Additives such as xylitol and maltitol aid remineralisation and can also affect bacterial gene expression and the oral microbiome ecology. Xylitol has been seen to cause a decrease in gtfB expression in *S. mutans* when xylitol chewing gum has been used for 12 months. GtfB is a glucosyltransferase, which helps *S. mutans* to adsorb to the dental pellicle via the synthesis of glucans (Bowen and Koo, 2011). This was seen to cause a reduction in the size and growth rate of *S. mutans* biofilms. This morphology change was likely due to a reduction in the production of sticky substances in response to the altered GtfB levels; a change in the quantity of *S. mutans* and a reduction in adherence of the colonies was also observed (Lee *et al.*, 2008).

Medicated chewing gums

The first medicated chewing gum (MCG) patent was filed in 1869, with the first MCG being launched in 1924 (Woodford and Lesko, 1981). Aspergum was a medicated chewing gum which contained aspirin. It was sold on the premise of providing pain relief for minor ailments from a sore throat to a mild headache. However, studies showed that four chewing gums would be needed to have the same effect as two aspirin tablets (Woodford and Lesko, 1981). MCGs started gaining traction in the 1980s with the sale of nicotine chewing gum (Russell, Raw and Jarvis, 1980), which became popular as a cigarette replacement and was prescribed by British doctors (Russell, Raw and Jarvis, 1980). This delivery system for nicotine is still popular today. In the 1990s, chlorhexidine chewing gums were being investigated (Jukka Ainamo, 1987; Tellefsen *et al.*, 1996). In 2006 CHewX, a chlorhexidine-containing chewing gum, began to be sold in Switzerland. CHewX was shown to be effective in plaque removal in adults and elderly people (Imfeld, 2006).

Effects of chewing gum on oral biofilms

The mechanical action and stresses of chewing gum will remove some bacteria from the teeth. However, very little is known about the effect of the shear stress on the biofilm when combined with the additional effect of the release of antimicrobial agents from the gum. By chewing gum that contains antimicrobial agents, it is expected that there will be a profound effect on the oral microbiome ecology. There will be a combined effect of three factors: the first being the mechanical forces that will disrupt the biofilm architecture and potentially shear off sections of the biofilm, the second is biofilm microcolonies may be entrapped in the gum and pulled off the tooth and gum surfaces, and the third potential effect is the antimicrobials released from the gum may be more effective at penetrating the disrupted biofilm.

Common oral antimicrobials

Chlorhexidine is the most well-known and commonly used oral antimicrobial; it is present in medicated mouthwashes. Chlorhexidine was developed in the 1940s and has since been used in many medical fields, including gynaecology, ophthalmology, and for the treatment of burns (Sajjan P, Laxminarayan N, Kar PP, 2016). It was first used in oral care in 1969 in an experimental study (Löe, 1969). Later, in 1976, the first chlorhexidine-medicated mouthwash trial was seen to alter the microbiome (RindomSchiostt *et al.*, 1976).

Depending on the concentration, chlorhexidine can be either bacteriostatic or bactericidal (Hennessey, 1973). Chlorhexidine is a positively charged molecule that specifically binds to the phosphate-containing molecules in the bacterial cell wall. This strong bond then leads to penetration of the cell wall and a reduction in its integrity. The weakened cell wall then allows the chlorhexidine molecules to penetrate the cell, where they precipitate in the cytoplasm and also co-aggregate with the phosphate-containing proteins within the cell, killing it (Jenkins, Addy and Wade, 1988; Kumar, 2017).

Chlorhexidine is a bisbiguanide; it has a central hexamethylene chain with a biguanide and 4-chlorophenyl ring on either side of the chain. This structure provides a positive charge on both ends of the compound (Mathur *et al.*, 2011).

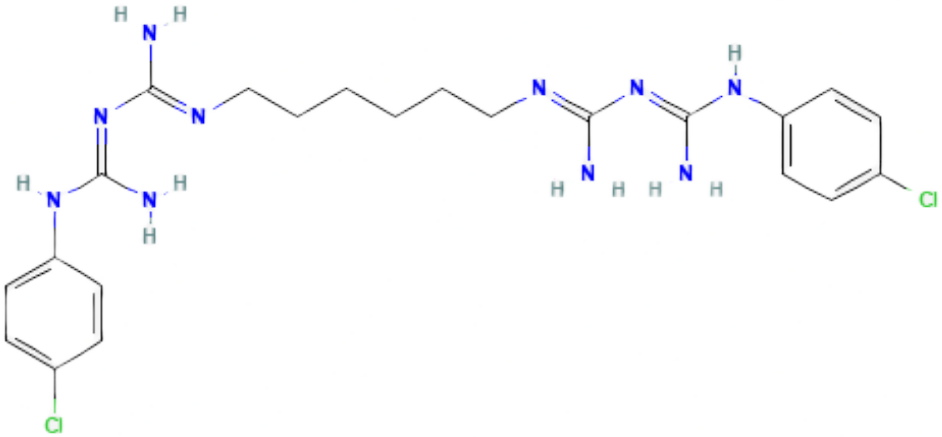


Figure 4: Molecular structure of chlorhexidine, reproduced from PubChem (PubChem, 2022).

Another common oral antimicrobial is CPC, a protective active compound used in mouthwashes. CPC has been shown to cause a reduction in viable bacteria when used as an oral rinse. The effect was greater than the fluoride rinse but slightly less effective than chlorhexidine (Sreenivasan, Haraszthy and Zambon, 2013). CPC is an amphiphilic quaternary compound with a positive charge

that aids in binding to negatively charged bacterial surfaces and denaturing them (Ioannou, Hanlon and Denyer, 2007).

Arginine and SnF_2 have also been used as additives to oral health care; this dentifrice has a protective role in the oral cavity rather than a bacteria removal role. It creates a protective layer over the enamel which results in enhanced resistance to acid dissolution (West *et al.*, 2017).

Natural product antimicrobials

Cranberry

Cranberries are a natural product that have been tested for their antimicrobial properties, especially as an oral antimicrobial compound. It has been tested as an active ingredient in mouthwashes to prevent S*treptococcal* colonisation. Current studies show that cranberry juice, and particularly its constituent nondialyzable material, reduce the adsorption of S*treptococcal spp.* to the tooth's pellicle. This reduction is caused by the cranberry constituents binding to the hydrophobic proteins on the cell surface (A. Yamanaka R. Kimizuka T. Kato K. Okuda, 2004). Deshmukh's group showed that cranberry extract has a similar effect on s*treptococcal* colonisation as chlorhexidine in a clinical test of mouthwash; however, this effect was achieved via two different mechanisms of action for the cranberry and chlorhexidine (Khairnar *et al.*, 2015).

Propolis

Propolis is a product produced by bees from the sap from trees mixed with their saliva. Propolis is thought to have antimicrobial properties; however, propolis varies hugely depending on where it was produced and what trees and flowers were used by the bees to create it. Propolis is made up of over 160 different components (Mirzoeva, Grishanin and Calder, 1997) and consists of 50% resin, 30% wax, 10% essential oils, 5% pollen, and 5% other substances (Monti M., Berti E, Carminati G, 1983). Across the varieties of propolis, the concentration of flavonoids and phenolic compounds varies, and this was found to be correlated with their antimicrobial activities (Górniak, Bartoszewski and Króliczewski, 2019). Some antimicrobial mechanisms include reduced or slowed biofilm formation (Wojtyczka *et al.*, 2013), membrane disruption (Cushnie and Lamb, 2005), RNA polymerase binding (Speciale *et al.*, 2006), and DNA gyrase binding in certain species (Plaper *et al.*, 2003).

Essential oils

Essential oils also have an antimicrobial effect, and they are used in commercial oral products such as Listerine. Essential oil mouthwashes have been shown to create a 78.7% reduction in bacterial viability (Ouhayoun, 2003; Quintas *et al.*, 2014). These mouthwashes use a combination

of many essential oils, often including thymol, eucalyptol, menthol, and methyl salicylate, in addition to alcohols, xylitol and sorbitol.

The mechanism of action for these essential oils is broadly to disrupt the bacterial cell walls and inhibit enzymatic activity (García-Salinas *et al.*, 2018). However, the specific details of how they work are less well-characterised. Thymol is one of the more well-investigated essential oils; the current views on its mechanism of action are based on the accumulation of thymol in the bacterial outer membrane. This accumulation weakens the membrane, allowing potassium ions to leak from the cell; thymol can then enter the cell, bind to other proteins, and cause ATP depletion (Nazzaro *et al.*, 2013).

Another essential oil with well-characterised antimicrobial properties is cinnamaldehyde, a compound from cinnamon powder. Cinnamaldehyde has bactericidal properties against oral bacteria, including *S. mutans* (He *et al.*, 2019). At sub-minimum bactericidal concentrations (MBC) in *S. mutans*, it can reduce biofilm biomass by increasing cell surface hydrophobicity and reducing EPS production via the downregulation of key genes (He *et al.*, 2019). It can also inhibit acid production and tolerance (Balasubramanian *et al.*, 2021). This antibacterial activity is centred on the membrane; however, it arises from several interactions both on the membrane and intracellularly. Cinnamaldehyde also inhibits GTP-dependent FtsZ polymerization, preventing bacterial cell division (Domadia *et al.*, 2007). In another Gram-positive facultative anaerobe, *Listeria monocytogenes,* cinnamaldehyde was shown to bind to membrane-associated enzymes and prevents the ATPase activity of the cell and reduces ATP synthesis within the cell (Gill and Holley, 2004).

1.6 Oral microbiology models

Surfaces

Biofilm Growth Systems, Static, Continuous, and Chewing models

The oral microbiome is a difficult environment to replicate *in vitro*. One common issue in studying oral microbiology is that often the *in vitro* models do not include ways to mimic eating and the associated fluctuations in glucose. This will affect how the bacteria grow and which bacteria grow based on their metabolic pathways. Some papers have shown that differing glucose levels cause diversity and pH to decrease, leading to certain species, such as *Streptococcus spp.,* increasing in abundance (Rudney *et al.*, 2012). A second limitation in many of these models is the lack of mechanical removal, which normally occurs by chewing either food or gum. *In vitro* models also

lack the pellicle present on the surfaces; this can be remedied by conditioning the surface in saliva or mucins to create a pellicle.

There are four main surfaces for studying oral biofilms *in vitro*: plastic, glass, hydroxyapatite, and bovine teeth (Darrene and Cecile, 2016). Plastic is one of the more commonly used surfaces to grow biofilms. This is because biofilms are often grown in well plates, which are conducive for screening as well as imaging through the transparent dish. Plastic, however, is not representative of the tooth surface, and therefore there may be changes in bacterial attachment due to the lack of glycoproteins. Growing biofilms on glass has the same pitfall as plastic, where the surface is not representative of the tooth's surface. However, one of the major positives to using glass is the ease of imaging on microscopes, such as the confocal, whereas plastic is not as optically clear for microscopic imaging.

Hydroxyapatite is a naturally occurring form of calcium apatite with the general molecular formular ($Ca_5(PO_4)_3(OH)$). Hydroxyapatite has a similar surface to human teeth, making it an ideal substrate for testing oral biofilms. Hydroxyapatite can be spray coated on glass slides or formed into solid discs compatible with commercially available flow cells and the Centre for Disease Control (CDC) bioreactor (Guggenheim *et al.*, 2004). However, because these discs are opaque, imaging is more complicated. The most representative *in vitro* surface option is bovine teeth. This allows the biofilm growth to be highly representative because all the normal glycoproteins found on teeth, which are used in attachment, are present. However, teeth will be harder to image because of their size, opacity, and curved shape.

A further improvement for growing oral biofilms is the use of dental plates (Dige *et al.*, 2009). This is where a coupon is inserted onto a tooth or held in place with a brace-like stent and then collected after an allotted period of time. This is the most realistic representation due to the oral glucose (typically 0.5-1 mg/100ml), flow, and mechanical changes that occur *in vivo,* which are hard to mimic *in vitro*. However, this requires ethics approval and numerous volunteers to account for the variation seen between individuals' microbiomes and eating habits and has a more complicated sample collection.

Biofilm Growth Systems

Bacterial species selection

There are three options for the selection of bacteria when completing experiments on the oral cavity. The first is to use a single species, usually an early coloniser of the oral cavity or a pathogenic oral bacterium. This is the easiest method for *in vitro* experiments due to the easier

growth conditions, no interspecies competition, and sourcing a single species of bacteria is straightforward.

The second is creating a microcosm, where a microcosm is defined as "a laboratory subset of the natural system from which it originates and from which it also evolves" (J W Wimpenny, 1997). This means the microcosm is the first evolution from a human sample. Biofilms created from the microcosm are *ex vivo* and are as similar to the oral microbiome, growth, and behaviour of an *in vivo* dental biofilm as possible within the lab setting. However, if the microcosm environment is not designed appropriately, it will not represent the *in vivo* community, affecting results (Koopman *et al.*, 2014).

The final method is to use a defined consortium with major plaque species or a mix of oral bacteria. This consortium would be manually created using selected species. This is easier to source than the microcosm and allows for specific inclusion/exclusion of species. This creates the opportunity to create a cariogenic community seen in diseases like dental caries (S K Filoche, K J Soma, 2007). However, optimisation would be needed to create an inoculum that allows all the incorporated species to thrive collectively. Often, in a consortium, certain species can outcompete others in a non-representative way (Saunders and Greenman, 2000).

Static reactor systems

There are two main systems used for the study of static growth of oral biofilms; these are the use of a Calgary device and the Zurich biofilm model. As a static model, they do not have the continual addition of fresh media; however, the media can be exchanged periodically. The Calgary device is a modified 96-well plate where the lid has pegs that extend down into the wells (Azeredo *et al.*, 2017). This is good for mimicking oral biofilm formation and stops gravity from accelerating sedimentation biofilm formation at the bottom; however, the pegs are small, on the order of 10 mm in length, and therefore are difficult to image or scrape; however, sonication can be used to remove the bacteria from the pegs.

The Zurich model is a protocol that uses hydroxyapatite discs in a 24-well plate; the biofilms are grown up over five days with media changes and dipped in a glucose broth three times a day to represent eating (Guggenheim *et al.*, 2004). This is a more representative static model as it uses a material that is similar to teeth, and the dipping would represent the daily increase in glucose levels seen during eating. An easier but less representative protocol is to use hydroxyapatite discs in a multi-well plate but not dipping in glucose three times a day (Koo *et al.*, 2003).

Continuous reactor systems

There are four continuous models used for studying oral biofilms that will be discussed, the first being the CDC Biofilm Reactor, a modified chemostat, as shown in Figure 5A (Reactor, 2017). The CDC reactor is an FDA-approved, continuously fed bioreactor (Azeredo *et al.*, 2017). The CDC Biofilm Reactor® is capable of holding up to 24 coupons at one time and continuously having new media pumped through at a specified rate which can match the average salivary flow in the mouth of 0.3 ml per minute (Humphrey, 2001). A positive advantage of the CDC Biofilm Reactor® is that all the coupons have the same exposure to the media, so they should all grow at the same rate. However, for oral biofilms, the quantity of media present is much more than normally found in the mouth. As well as being approved by the Food and Drug Administration (FDA), the Environmental Protection Agency (EPA) has brought out a method for using the CDC Biofilm Reactor® which is being included in the new FDA guidelines (US EPA, 2017). This shows this method has had the method and the repeatability of the biofilm grown on the coupons verified.

The second model is the Bio-inLine® Biofilm Reactor, which is a continuous flow reactor where the media runs from one end to the other running over the coupons in the middle, as shown in Figure 5B (BiosurfaceTechnologies, 2019). It is a modified form of the Robbins device. Due to the Bio-inLine® Biofilm Reactors' size (½ inch square channel), it requires less media and is more bench friendly than a CDC Biofilm Reactor®. One problem is that the coupons will receive varying amounts of nutrients based on how far down the reactor they are placed. The upstream biofilm removes nutrients and produces waste products which then flow over the later discs.

The third model is a flow cell; they are a much smaller version of a continuous culture system which allows for a highly controlled environment (Crusz *et al.*, 2012; Azeredo *et al.*, 2017); and is seen in Figure 5C (Crusz *et al.*, 2012). It can also be used with real-time imaging, allowing for images on biofilm formation to be collected, which is much harder with the CDC and inline reactor as the coupons would have to be removed and reinserted. Flow cells are generally much smaller, therefore use less media; however, at this size they would not be able to hold hydroxyapatite on it and, therefore would be less representative of teeth.

Another continuous flow model is the Constant Depth Film Fermenter, scraper blades are used to maintain a constant depth on the discs holding the samples; a schematic is shown in Figure 5D (Pratten, 2007). The removal of excess biofilm mimics the movement of the tongue over the teeth, making it more representative of *in vivo* conditions. This model is often used to study biofilm structure and the effects of antimicrobials on biofilms since it is capable of producing 15 replicate biofilms (M Mei et al., 2017). There are many other models used including 3D printed

and microfluidic cells, however, for brevity they will not be discussed in this literature review, more information can be found in (Brown *et al.*, 2019).

a)

b)

c)

d)

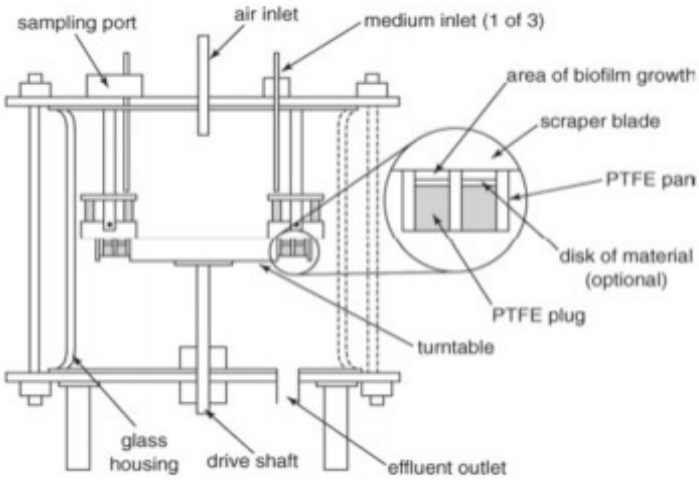


Figure 5: Images showing a: the centre for disease control bioreactor, b: bio-inline reactor, c: the microbial flow cell, and d: the constant depth film fermenter. All images reproduced with the permissions of the authors; a and b: Biosurface Technologies 2017 and Biosurface Technologies 2019, c: Crusz et al., 2012, d: Pratten, 2007.

Mechanical chewing models

There is one common way to mimic chewing; the ERWEKA model; it is used to assess the effect of chewing on the rheology of the gum and the release of flavours. The ERWEKA model is a machine designed to mimic chewing (shown in Figure 6a). It is the most realistic chewing machine and is capable of holding the media around the coupon with a lever, which depresses down onto the coupon, as shown in Figure 6a (*Chewing Gum Tester DRT - ERWEKA GmbH*, 2019); the ERWEKA also is capable of revolving as it moves up and down. However, this system is very expensive, so it is not commonly used in academic research experiments but is used more in industry for experiments and product testing.

The Instron Electropuls E1000 (shown in Figure 6b) is a potential novel chewing machine, it is an indenter that is capable of applying pressure to a hydroxyapatite disc for an extended period at a defined rate and mimics "chewing" with an up and down motion. It works in a similar way to the ERWEKA machine; however, it is more often used to test materials and objects rather than "chewing".

a)

b)

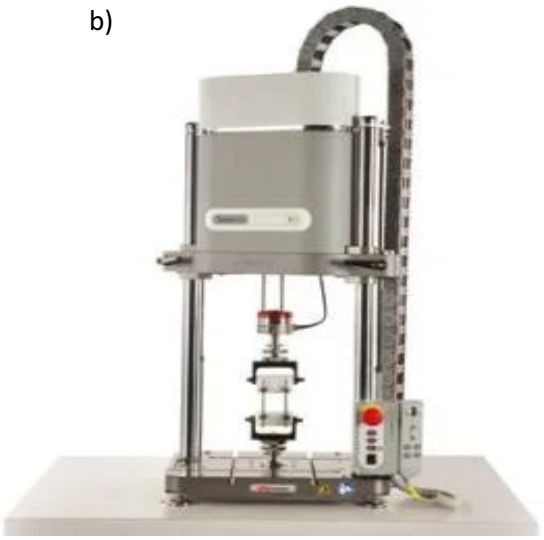


Figure 6: a: the ERWEKA chewing machine ("Chewing gum tester DRT – ERWEKA GmbH" n.d.) b: shows the Instron Electropuls E1000 (Designed, 2014).

Project aims

The work presented in this thesis aims to provide a new understanding of the interaction between the mechanical forces occurring within the oral cavity and the oral microbiome and to improve understanding of the impact of combined mechanical disruption with a simultaneous release of antimicrobial agents on simulated biofilm plaque.

1) To develop a novel, *in vitro* oral biofilm model that incorporates a simple mechanical chewing mechanism through oscillating pressure (Chapter 2).

2) To explore the efficacy of eight selected antimicrobial agents against *Streptococcus mutans* in planktonic and biofilm states and to identify the top candidates with the potential for use in oral disease reduction and prevention (Chapter 3).

3) Building on the previously gained knowledge from the literature search and Chapter 3 to develop a range of antimicrobial chewing gums and characterise their release rates (Chapter 3).

4) To characterise the effects of the combination of antimicrobials and mechanical forces on *Streptococcus mutans* biofilms and determine if there is a potential additive or synergistic effect (Chapter 3).

5) To use the novel model and collected *ex vivo* saliva-based inoculum to analyse the effects of key antimicrobials and chewing on biofilm culturable counts, viability, and structure (Chapter 4).

6) To optimise the extracted DNA yield from *ex vivo* oral biofilms for the study of the oral microbiome (Chapter 4).

Chapter 2:

Design and optimisation of an oral model which incorporates the mechanical action of chewing.

"All models are wrong, but some are useful".

George E. P. Box

Chapter 2 Development of a novel oral biofilm model which incorporates the mechanical actions of chewing.

2.1 Introduction

The work described in this chapter aims to develop a novel model for oral biofilms which can incorporate the mechanical actions of chewing. Many oral microbiology experiments which investigate chewing gums are done *in vivo* (Al-Ahmad *et al.*, 2010; Klug *et al.*, 2016), and of those done *in vitro,* most do not include a chewing stage (Ceri *et al.*, 1999; Guggenheim *et al.*, 2004).

This project aims to investigate the effect of chewing gum on the oral microbiome and characterise the combined effects of mechanical chewing in the presence of antimicrobials. For these experiments, a model needed to be developed to allow for the discovery of how the oral biofilms interact with chewing gums' mechanical forces in addition to antimicrobial exposure.

Three main methods to investigate oral biofilms will be discussed; the first is the Zurich model, developed by Guggenheim et al in 2001. It is a multispecies model which uses hydroxyapatite discs in a static well plate (Guggenheim *et al.*, 2001). This model has been used for oral microbiology extensively and has been cited 243 times according to Web of Science, the latest as recently as January 2023, showing its continued use (Guggenheim *et al.*, 2004; Bloch *et al.*, 2017; Murugkar *et al.*, 2023). The species used are *Actinomyces naeslundii*, *Fusobacterium nucleatum*, *Streptococcus oralis*, *Streptococcus sobrinus* and *Veillonella dispar*. These bacteria are grown in modified fluid universal medium, supplemented with Sorensen's buffer for pH correction. The bacteria are added to a 24-well plate with a sintered circular hydroxyapatite disc in each well and left to grow from 0.5 hours to 64.5 hours in an anaerobic chamber.

The second common growth system is to use a CDC bioreactor; it is FDA approved and can run with either basal mucin media or supplemented BHI media (Rudney *et al.*, 2012; An *et al.*, 2022). For the growth of *ex vivo* inoculum, the CDC bioreactors are run aerobically at 37°C, using hydroxyapatite discs. Rudney et al use a media input of 17 ml/min, whereas An et al use a much lower input rate of 0.5 ml/min. Rudney et al also use a batch stage after adding the initial inoculum; no fresh media is added for the first 24 hours, whilst An et al use a method based on the American Society for Testing and Materials standard E2562-07.

The third commonly used method for studying oral biofilms is to ask participants to wear an oral splint which can hold either hydroxyapatite, metal or bovine tooth discs. This is one of the most

representative models, as the biofilms are formed directly in the oral cavity using the bacteria present. These splints are worn for between 24 and 72 hours; however, 48 hours was determined to have the best biofilm formation (Huang *et al.*, 2021). To protect the biofilm growth these splints are removed for eating and tooth brushing, which is done without toothpaste (Becker *et al.*, 2021). Whilst this model is arguably the most relevant, it is one of the most complex due to using participants, meaning ethical approval must be obtained, and the use of these biofilm discs for multiple experiments would require many participants or repetitive use of the same participants. This model also creates biofilms based on one person's oral microbiome, which, as discussed earlier, is as specific to a person as their fingerprint, and may thus affect the results gathered. Each person's oral microbiome is affected by their oral hygiene methods, diet and lifestyle. Therefore, in other models, pooling is often used when creating an *ex vivo* microcosm to create a diverse community, such as in (An *et al.*, 2022).

These three models are used to study oral biofilms; however, they do not include chewing forces. My project is aimed at determining the effects of antimicrobial chewing gums and the potential synergism between chewing and antimicrobials, making the chewing action essential.

One model which incorporated a chewing action was developed by Wessel *et al.*, (Wessel *et al.*, 2015). This model uses a sterile glove finger with a 1.5-gram piece of chewing gum and 200 µl of bacterial suspension inside. The finger of the glove with gum in it is placed in a water bath at 37°C and manually pressed together for five minutes. This was completed with four individual bacterial species: *Streptococcus oralis*, *Streptococcus mutans*, *Streptococcus mitis* and *Actinomyces naeslundii*. Whilst this model does incorporate chewing actions, it is used on planktonic bacteria, which is representative of the salivary microbiome but not of the areas of the oral cavity which have biofilms present, which is where most oral diseases stem from. Bacteria in the planktonic and biofilm states are very different in regard to their metabolism, gene expression and antimicrobial resistance. To investigate fully if an antimicrobial would have a positive effect on the oral cavity, both planktonic and biofilm states should be investigated.

A model used for testing chewing gums is the ERWEKA, Figure 6A. The ERWEKA is a machine used to test the release of compounds from chewing gums and is not currently used in conjunction with biofilms. It uses a vertical and angular rotation to mimic the chewing actions, from above and below the sample, at a set rate and pressure. Whilst this is the gold standard for chewing experiments in industry, its use was not possible in an academic setting.

2.2 Chapter aims

The first aim of this chapter was to develop a novel, *in vitro* oral biofilm model that incorporates a simple mechanical chewing mechanism through oscillatory pressure.

Then to investigate and compare different growth systems and chewing mechanisms.

The final aim was to characterise the effects of oscillatory pressures from chewing on *Streptococcus mutans* biofilm viability, culturable counts and architecture.

2.3 Methods

2.3.1 Bacterial strains and culturing

The bacterial strain used was *S. mutans UA159* (American Type Culture Collection (ATCC), 700610). *S. mutans* was chosen due to the important role it plays in early colonisation and dental caries. *S. mutans* was cultured on BHI agar (Merck) overnight at 37°C in a 5% CO_2 incubator to confirm purity. The media used was BHI, as recommended by the ATCC for the growth of *S. mutans*. A single colony was selected and inoculated into 10 ml of BHI broth (Merck) and incubated overnight at 37°C in a CO_2 incubator to create an overnight culture. Glycerol stocks were created by the addition of 750 µl of the overnight culture to 750 µl of 50% glycerol (ThermoFisher) in a cryovial and were stored at -80°C.

2.3.2 Growth system investigations

2.3.2.1 *S. mutans* grown in a Zurich-based model system

A streak plate and overnight culture was grown, as previously stated, of *S. mutans* wild type. Sterile hydroxyapatite discs (BioSurfaces Technologies) were added to a 12-well plate with 1 ml of overnight culture and 1 ml of 1/5th strength BHI broth made up with phosphate-buffered saline (PBS). The plate was placed in a 37°C CO_2 incubator for 72 hours. Every 24 hours, 1 ml of broth was removed from the well, and 1 ml of fresh 1/5th BHI broth was added gently down the side of the well. After 72 hours, all broth was removed from each well and the discs washed in 2 ml of PBS (Thermo Fisher) using a rocking motion.

The discs were then either scraped using a cell scraper, serially diluted 10-fold and then spot plated or directly LIVE/DEAD stained for confocal imaging. LIVE/DEAD BacLight Bacterial Viability Kit (Life Technologies) contains SYTO9 and propidium iodide to stain the live cells green and the dead cells red. SYTO9 can pass through the membranes of bacterial cells, whilst propidium iodide is a much larger molecule which can only pass into cells with disrupted membranes, which indicates the cell is dead. For the cell scraping, the wash was removed, and 1 ml of fresh PBS was added directly onto the biofilm. A cell scraper (SLS) was then scraped over the disc for 30 seconds. The PBS was washed over the disc with a pipette three times before 200 µl was removed and added to a 96-well plate for spot plating. This was done by completing a 10-fold serial dilution, seven times sequentially in PBS, by adding 20 µl to 180 µl of PBS to produce final concentrations ranging from 100% to 0.00001%. These were spot plated onto a BHI agar plate with three spots of 10 µl and incubated overnight at 37°C in a CO_2 incubator.

For the confocal laser scanning microscopy (CLSM), a LIVE/DEAD stain was prepared by creating a stock of 2 µl of propidium iodide and 2 µl of SYTO9 per 1 ml of PBS. The discs were then exposed to the stain for 10 minutes before washing them with PBS. The discs were then set in a hard-set resin to allow them to be inverted without altering the biofilm structure due to the CLSM being an inverted model. The discs were partially dried before 40 µl of Mowiol hard set resin (Sigma-Aldrich) was added to the top of the disc, then heated at 45°C for 30 minutes before a coverslip was placed on top and left to set.

Samples were imaged using the inverted Leica SP8 confocal laser scanning microscope. A 63X oil immersion lens was used with Argon and diode-pumped solid-state (DPSS) lasers, scanning sequentially at 1 µm intervals. These lasers were set at 488 nm and 561 nm, respectively to excite the dyes. Three images were taken on each disc, in identical places on each sample to avoid bias; the first image was in the centre of the disc and then one on either side. Initially, to get to the correct focus, a fluorescein isothiocyanate (FITC) filter was used on the first sample site. Images were obtained using the Leica Application Suite X (LAS X) software.

2.3.2.2 *S. mutans* grown in a CDC bioreactor system

A CDC bioreactor (BioSurfaces Technologies) containing hydroxyapatite discs and 350 ml of BHI was sterilised and set up on a stirring hot plate at 37°C, stirring at 60rpm, with a 0.3 ml/min flow rate. The temperature of the media was measured during a pilot experiment to ensure the correct internal temperature was being reached. This CDC bioreactor setup is shown in Figure 7. As described before, an overnight culture was created of *S. mutans*; and 1 ml of the overnight culture was added to the CDC bioreactor. This was then run for 72 hours before the discs were removed from the reactor and processed in a microbial safety cabinet. The CDC bioreactor is run aerobically, however, there is no active air input into the reactor the only gas exchange is passive through a 0.2 µm filter. It is known that the presence and concentration of oxygen effects bacterial metabolism (Carlsson, Iwami and Yamada, 1983). Quantifying the level of oxygen in the bioreactors could provide useful insights. These discs were imaged and scraped as previously described.

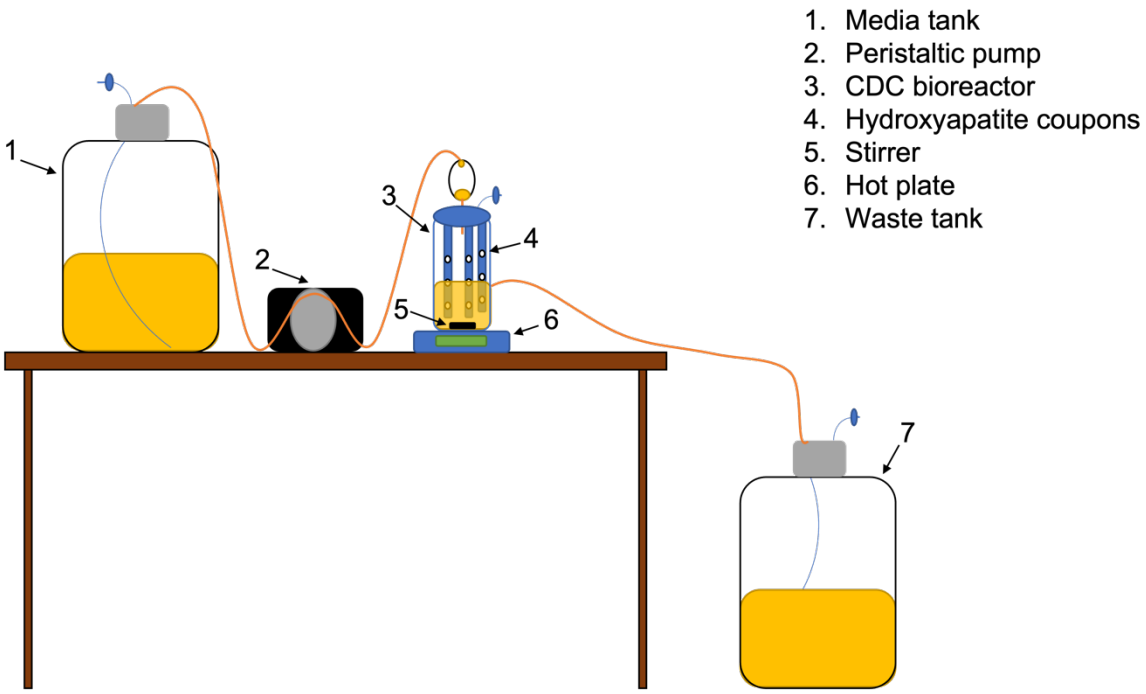


Figure 7: Schematic of a Centre for Disease Control (CDC) bioreactor.

2.3.3 The addition of a chewing mechanism to the testing model

2.3.3.1 Manual chewing method

S. mutans biofilms were grown using the Zurich-based model system (as described in 2.3.2.1). After 72 hours, the discs were moved to a sterile 12-well plate, and 1 ml of PBS was added. A 2-gram piece of chewing gum was placed on top of the hydroxyapatite disc. Using sterile gloves, a finger was pressed down onto the gum above the biofilm at approximately 90 presses per minute for 15 minutes. These biofilms were then scraped, serially diluted, and plated out on a BHI plate as previously described.

2.3.3.2 Development and optimisation of an indenter mechanical chewing method

S. mutans biofilms were grown using the Zurich-based model system (as described in 2.3.2.1). After 72 hours, the discs were moved to a sterile 12-well plate. An E1000 Electropuls indenter was used to mimic the mechanical actions of chewing.

A collar, plunger and paddle were 3D printed out of polylactic acid; the collar holds the chewing gum in place over the disc, which is shown in Figure 8. The plunger allows the indenter to apply force to the chewing gum with the new collar in place, and the paddle lifts the disc out of the

collar after the chewing has occurred. These three 3D-printed pieces slot together with the hydroxyapatite disc and chewing gum inside.

A 2-gram piece of chewing gum base was placed on top of the hydroxyapatite disc, and the indenter was set to chew at 1.5 Hz and 90 N in a sine wave oscillation, as imaged in Figure 9 and Figure 10. To keep the force constant at 90 N, the displacement distance of the indenter arm was allowed to vary. These biofilms were then scraped, serially diluted, and plated out on a BHI plate as previously described.

To improve the representivity of the system to the oral cavity, the 3D-printed parts were placed in a 50 ml beaker; this allowed for 20 ml of PBS to be added to the system for the duration of the chewing period.

A piece of PA6 Nylon mesh was used to separate the chewing gum from the disc. The mesh was cut to a one-inch square and placed over the chewing gum. The use of mesh was tested to prevent the chewing gum from sticking to the hydroxyapatite disc and provide the antimicrobials with increased contact with the biofilms. The E1000 indenter was then run as previously described. Hereon we use the terms “chewing” or “chewed” to refer to specimens that have been subjected to the manual and mechanical chewing methods.

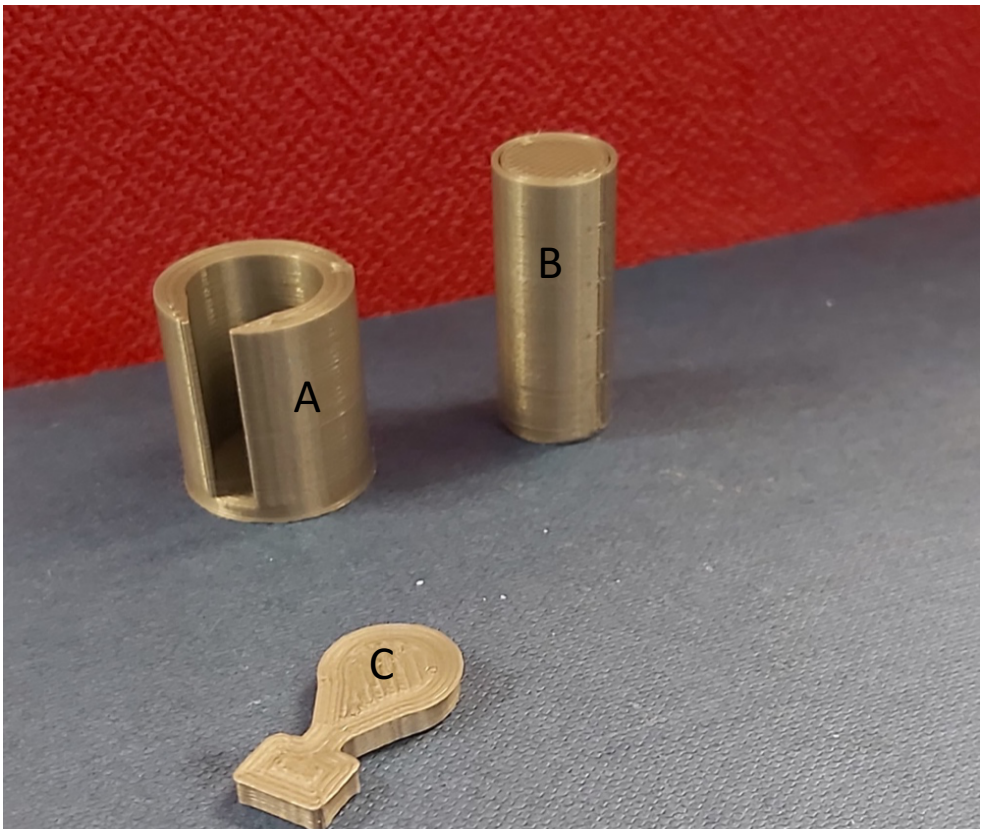


Figure 8: Photograph of the 3D printed collar (A), plunger (B) and paddle (C) accessories for the E1000 Electropuls indenter.

Figure 9: Photograph of the E1000 Electropuls indenter and accompanying computer.

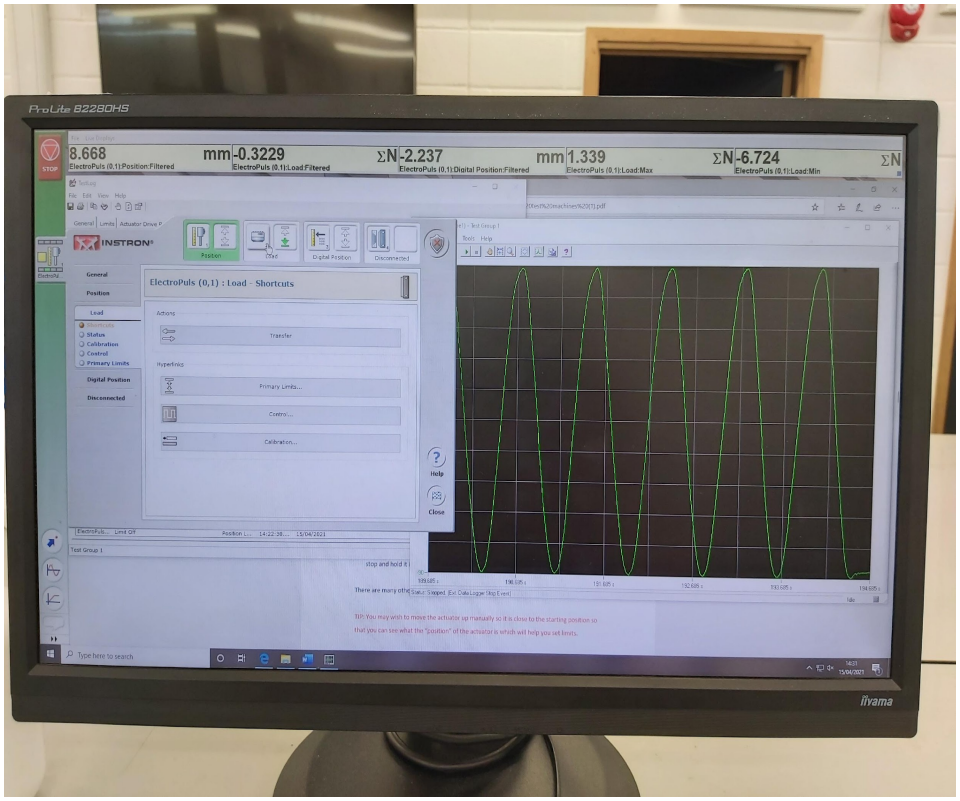


Figure 10: Photograph of the E1000 Electropuls indenter screen showing the sine waves that the indenter is following, representing the force applied over time.

2.3.3.3 Statistical analysis

The statistical tests used in this chapter are stated in the figure legends of each figure, including an unpaired T-test, standard deviation, and analysis of variance (ANOVA). GraphPad Prism 9 was used to run the statistical analyses. Differences were considered statistically significant for $P<0.05$. For bacterial culturable counts, the data was log-transformed, and then the mean and standard deviation was plotted; this was done to normalise the data.

2.4 Results

2.4.1 Growth system investigations

2.4.1.1 *S. mutans* grown in a Zurich-based model system

The first step for developing the model was to characterise the growth system. Initially, the system used for static growth was a Zurich-based model. The main differences from the published Zurich method were the use of one species only and the simplified media. It used hydroxyapatite discs in a 12-well plate, grown statically with daily media changes. The change in the well size, compared to the Zurich model, allowed for easier disc scraping using a cell scraper. Figure 11A and 11B show laser scanning confocal microscope images of the 3-day *S. mutans* grown in a Zurich-based model. In Figure 11A and B, a mixed population of viable and non-viable bacteria was observed, with an average of 67.5% of cells stained green. Both images show a biofilm with a flat structure measuring over 20 µm, with distinct cells.

2.4.1.2 *S. mutans* grown in a CDC bioreactor system

The second growth system to be compared was the CDC bioreactor. Figure 12 shows *S. mutans* grown in a CDC bioreactor over seven days. There was no significant difference in the number of culturable cells over the seven days of growth, P=0.24. Figure 11C and D show laser scanning confocal microscope images of the 3-day *S. mutans* grown in a CDC bioreactor system. In Figure 11C and D the viability of the biofilm was high, with an average percentage of cells stained green of 86%. The biofilms imaged are thicker than those in Figure 11A and B, measuring over 40 µm. This biofilm has an undulating structure with areas of varying thickness.

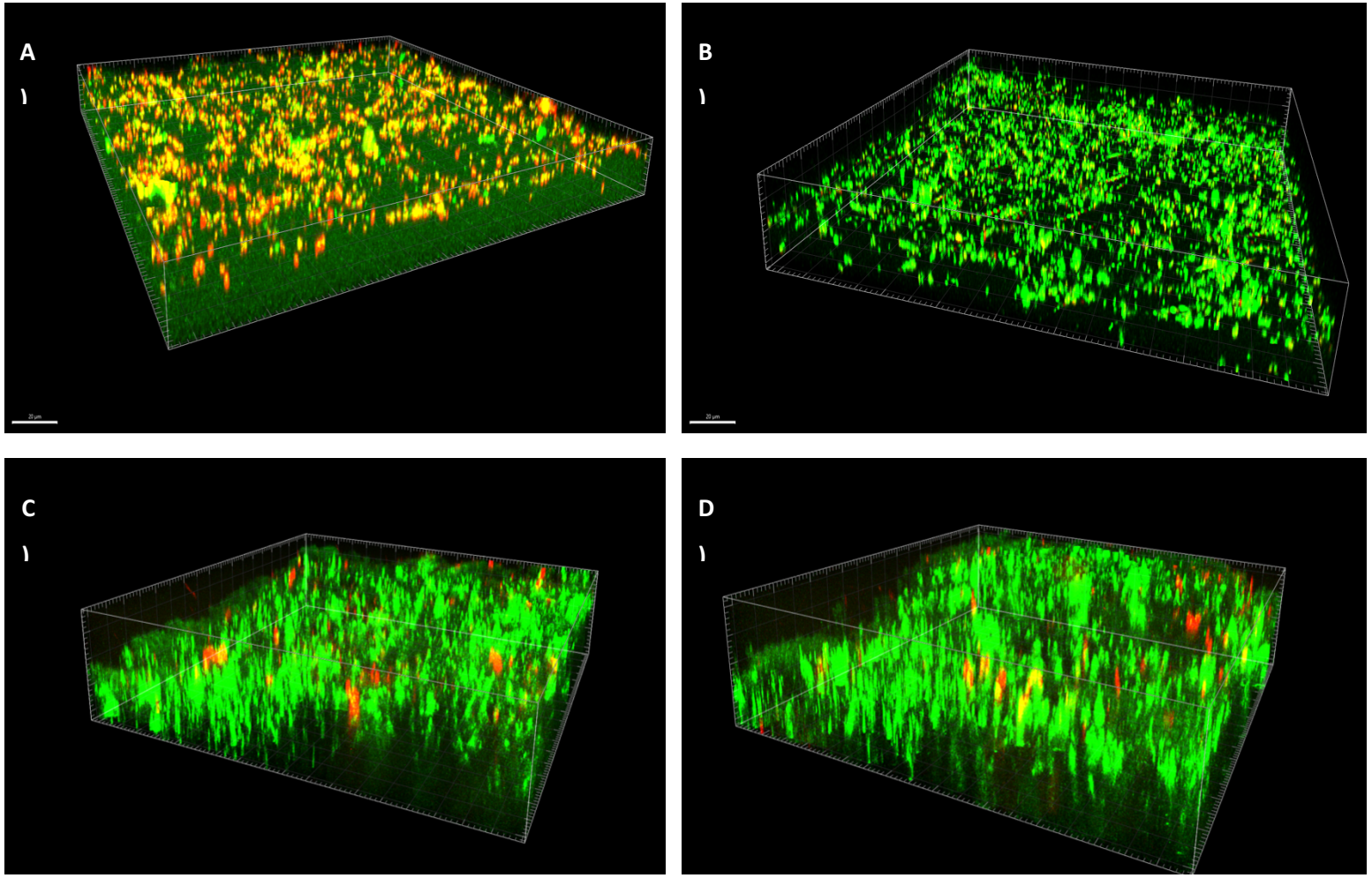


Figure 11: Laser scanning confocal microscopy images of a 3-day *Streptococcus mutans* biofilms grown on hydroxyapatite. A and B are grown using the Zurich based model. C and D are grown using the centre for disease control bioreactor model. These biofilms are stained with LIVE/DEAD stain, staining the viable cells green and the non-viable cells red. The scale bar in the bottom right indicates a 20 μm length.

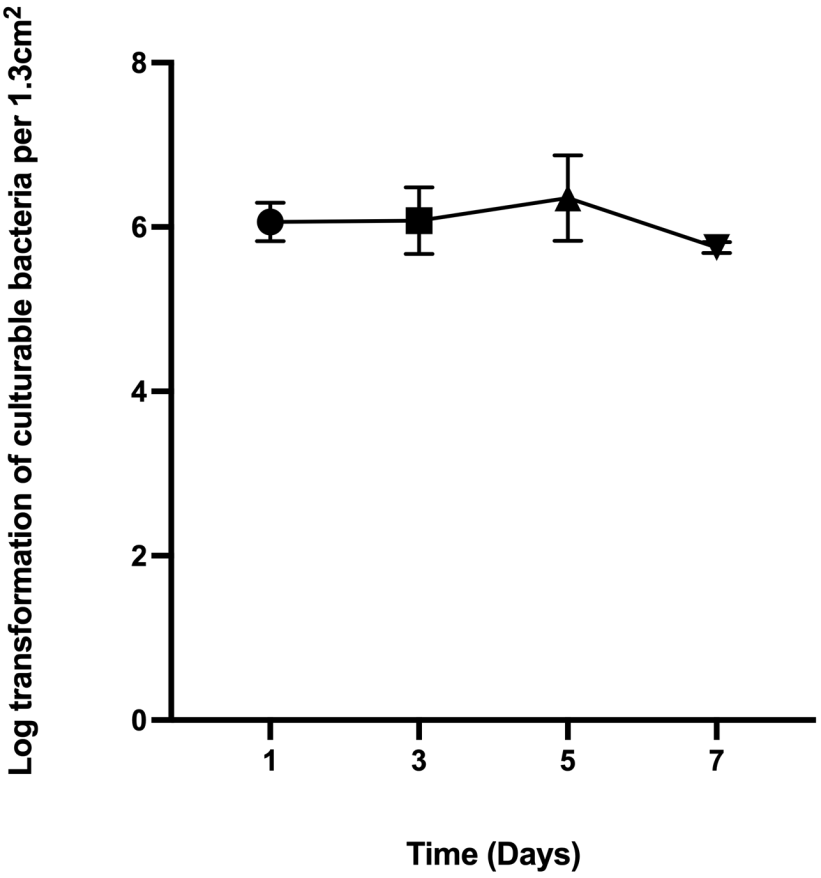


Figure 12: Graph to show the log-transformed number of culturable Streptococcus mutans over the first seven days of growth in a Centre for Disease Control bioreactor on hydroxyapatite discs. The mean of N= 3 plotted with standard deviation error bars.

2.4.2 The addition of chewing mechanisms to the model

2.4.2.1 Manual chewing method

To create a biofilm chewing model, the first step was to modify the manual chewing method from the Wessel method for chewing planktonic bacteria (Wessel *et al.*, 2015). The discs were "chewed" using a pressing motion whilst wearing a sterile glove, this modification allows for the investigation of biofilms attached to a surface, and the inclusion of PBS increases the representivity of the model by mimicking the presence of saliva.

To investigate how the growth system affects the efficacy of chewing, Figure 13 shows the number of culturable *S. mutans* bacteria after growing for three days in either a CDC bioreactor or in a static Zurich-based system. The controls are shown in grey, whilst the finger-chewed numbers are shown in beige. The CDC bioreactor control discs had 2.5 log fewer culturable bacteria than the statically grown discs, and there was no significant difference between the control and the

chewed value. In the static growth system, a significant decrease was observed when comparing the control and chewed values. The 2-log reduction in viability in the static growth system was much greater than the reduction seen in the CDC bioreactor culturable counts.

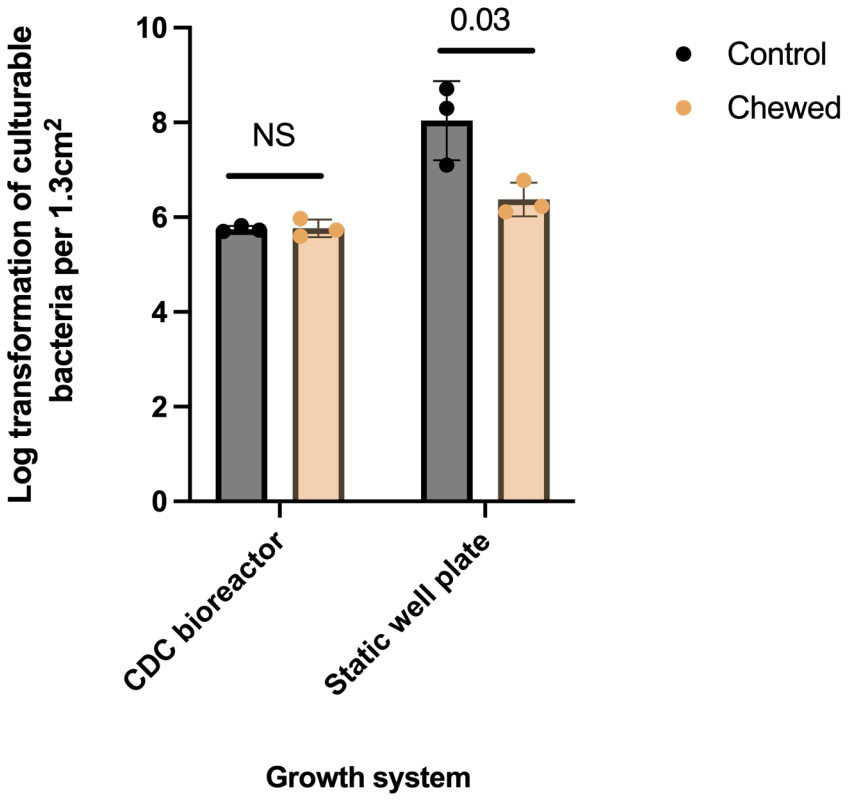


Figure 13: The log transformation of the number of culturable *Streptococcus mutans* bacteria after 3 days of growth in the centre for disease control bioreactor and 3 days of growth in the Zurich-based static model in grey. Discs from both the growth systems were chewed using a modified Wessel method for 15 minutes; their culturable counts are shown here in beige. The mean is plotted, N=3 with standard deviation. T-test completed for each set; P-value is shown.

The next step was investigating how the chewing affects biofilm architecture, Figure 14 shows the CLSM images of the 3-day *S. mutans* grown in a Zurich-based model, which were then exposed to manual chewing, using the modified Wessel method. Both images show a minimal quantity of individually stained cells, with a greater proportion of non-viable, compared to Figure 11A and B; however, there was still a large amount of background autofluorescence.

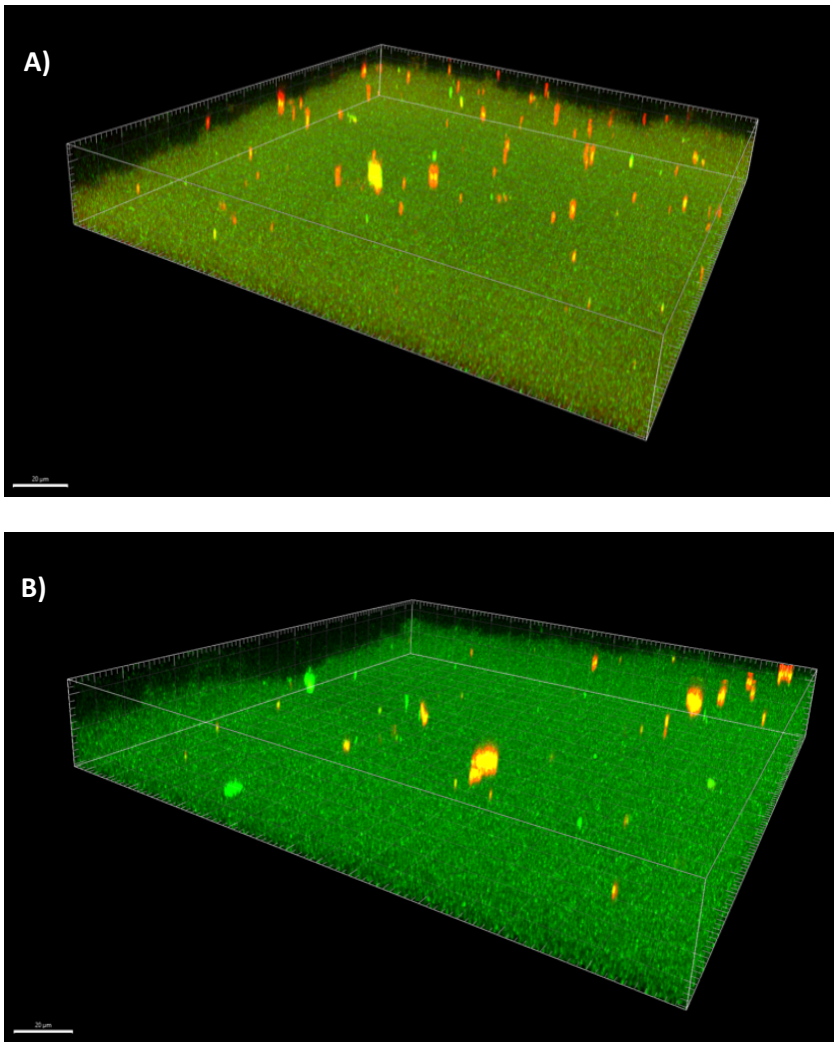


Figure 14: Two confocal laser scanning microscopy images of a 3-day *Streptococcus mutans* biofilm, grown using the Zurich based model, which have been manually chewed using the modified Wessel method, for 15-minutes. These biofilms are stained with LIVE/DEAD stain, staining the viable cells green and the non-viable cells red. The scale bar in the bottom right indicates a 20 m length.

2.4.2.2 Optimisation of the mechanical chewing method

Developing a mechanical method for chewing biofilms could be beneficial for the mass processing of samples. The second chewing method incorporated a mechanical chewing machine, the E1000 Electropuls indenter. Figure 16 shows the indenter pressing on a hydroxyapatite disc with gum over it. The gum has splayed out over the disc reducing its contact with the disc. This caused the disc to be in direct contact with the indenter arm. To improve the connection between the indenter, chewing gum and disc, three items were 3D-printed, as shown in Figure 8. The addition of the collar held the chewing gum in place for the full 15-minute chewing period, whilst the paddle allowed for the hydroxyapatite disc to be removed easily, and the plunger helped the indenter arm reach the disc after the addition of the collar.

Figure 15 compares the number of culturable *S. mutans* on a 3-day biofilm with no chewing, chewing from the E1000 Electropuls indenter and manual chewing. A significant reduction in culturable bacteria was observed from both the mechanical (2-log) and manual chewing (1.5-log). However, there was no significant difference between the mechanical and manual chewing.

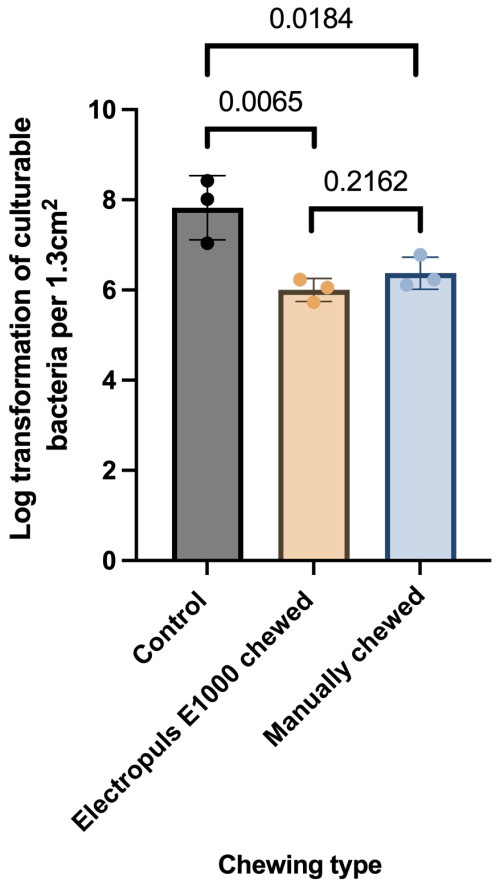


Figure 16: Photograph of the E1000 Electropuls indenter chewing the chewing gum on a hydroxyapatite disc.

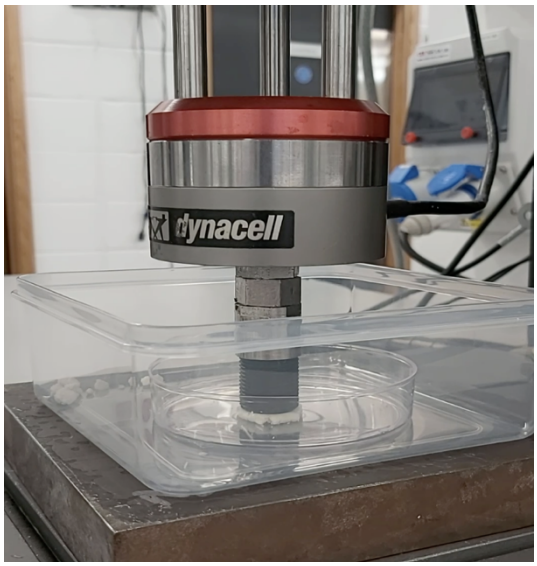


Figure 15: Graph showing the log transformations of the number of culturable 3-day biofilm, *Streptococcus mutans* bacteria, with no chewing, chewing from the Electropuls E1000 indenter, and manual chewing. These biofilms were grown using the Zurich based model. N=3 mean plotted with standard deviation error bars.

2.5 Discussion

2.5.1 Comparing different growth systems for growing *Streptococcus mutans* biofilms

This work developed and examined two model systems that allow for the growth and evaluation of oral biofilms. The Zurich-based model had the advantage that it allowed for a quick set-up of an oral biofilm model, compared to the CDC bioreactor. It was also an easier model to identify and detect contaminated samples early, thereby saving experimental time. However, during the growing phase of the model, there was more daily interaction with daily media changes. In contrast, the CDC bioreactor has a more intricate and laborious setting up phase; however, once running needs no interaction and can produce many replicates per run, with 24 discs per reactor.

In Figure 12, a steady level of culturable bacteria was seen across the first five days for samples prepared in the CDC bioreactor. At day seven, there was a decrease in culturable bacteria present, though this decrease was not significant. The number of culturable bacteria observed in Figure 12 are lower than anticipated for a standard *S. mutans* growth in a CDC bioreactor. To ensure all of the bacteria are being removed from the discs visualising the discs using microscopy would show any remaining bacteria. Another improvement would be to sonicate the samples after cell scraping to ensure any chains formed by the *S. mutans* are broken up into individual bacterial cells.

When growing *S. mutans* in the Zurich-based model, a higher culturable count was observed compared to the CDC bioreactor, as observed in Figure 13. This increase may be due to gravitational effects on the biofilm; since there were no shear stresses present in the Zurich-based model, the planktonic bacteria are capable of settling on the hydroxyapatite discs and forming biofilms.

Figure 11 shows CLSM images of the 3-day *S. mutans* grown in a Zurich-based model (A and B) and 3-day *S. mutans* grown in a CDC bioreactor (C and D). The images of the Zurich-based model biofilms have a level of variability in the presence of dead bacteria; this shows the variation within the system. The biofilms grown in the CDC bioreactor were thicker than the biofilms grown in the Zurich-based model; this contends with the CFU counts observed in Figure 13, which show a higher level of culturable bacteria in the static model. It was hypothesized that the increased thickness but not culturable count is caused by the presence of shear flow on the biofilm during the growth phase. The presence of shear flow increases EPS production up to a point, after which the shear force exceeds the EPS protective capability, and it starts to erode the biofilm (Ai *et al.*, 2016). There was also a reduction in clarity of the images taken in Figure 11C and 11D compared

to Figure 11A and 11B; it was hypothesised that this was due to the increase in EPS present, affecting the stain emission readings.

2.5.2 The addition of chewing mechanisms to the model

When using the developed modified Wessel method of chewing gum to chew biofilms, a significant reduction in the bacterial culturable count was observed, as shown in Figure 15. This was also observed in experiments carried out earlier (Figure 13); however, only in relation to the statically grown biofilms. The number of bacteria removed by chewing was $2.4X10^8$ for biofilms grown in the Zurich based model, this was similar to the number of bacteria removed during chewing by (Wessel *et al.*, 2015) at "around 10^8".

In Figure 14, two CLSM images of 3-day *S. mutans* biofilms are shown. They were grown using the Zurich based model, then manually chewed for 15 minutes using the modified Wessel method. There was a large reduction in the number of visible stained cells in Figure 14A and 14B compared to Figure 11A and 11B. The images in Figure 14 shows a high level of background noise, this could be attributed to EPS or hydroxyapatite disc autofluorescence. These may have more of an effect in Figure 14 due to the biofilm being significantly sparser than in Figure 11.

One of the modified Wessel method models' benefits was that it could be completed in a microbiological safety cabinet, providing more of a sterile environment for experiments to be conducted in, compared to a laboratory bench. This modified Wessel method was also beneficial as it does not require any extra equipment and therefore, can be repeated without requiring expensive and specialized equipment. One disadvantage to this model was the variation that could occur due to human error; when manually pressing the discs over a fifteen-minute period, the pressure and rate may change. These variations may be magnified if a large number of discs are processed at the same time.

This manual chewing method was tested on *S. mutans* biofilms grown in both the CDC bioreactor and statically. The biofilms from the CDC bioreactor were observed to be more resilient to the oscillatory forces of chewing, as shown in Figure 13. One hypothesis was that the biofilms are exposed to a higher level of shear stress in a CDC bioreactor due to the continuous flow. This leads to a thicker biofilm with more EPS present, creating a more resilient biofilm. Another difference between the two models is the increased air-liquid interface. The static model has a much higher gas transfer than the continuous CDC bioreactor, this creates a difference in the environment and may affect bacterial culturability.

It is hypothesized that biofilms from the oral cavity will land in between these two models; over the day, the surfaces in the oral cavity are covered in a film of saliva, similar to the Zurich-based model. However, throughout the day, food and drink will be consumed, increasing the salivary flow and increasing the shear forces present in a manner similar to the CDC bioreactor model.

Initial attempts at using the E1000 Electropuls indenter showed that the chewing gum would push the disc around, exposing the disc to the indenter arm. To remedy this, three parts were 3D printed, the collar to hold the gum over the disc, the paddle to help get the disc out of the collar after chewing and the plunger to help the indenter arm reach the chewing gum. This was successful in keeping the chewing gum above the disc, with only a small amount coming out of the front. The next step was to use 20 ml of PBS to help keep the chewing gum wet and replicate oral conditions. To do this, a 50 ml beaker was used to keep the PBS around the disc and not spread out, as occurred when using a petri dish.

A significant reduction in the number of culturable bacteria was seen when exposed to chewing via the E1000 Electropuls indenter compared to the control, as shown in Figure 15. This reduction in culturable bacteria was significant compared to the control; however, there was no significant difference between the mechanical and manual chewing. This indicates that both the manual and mechanical chewing methods had a similar effect and are both capable of reducing oral biofilms.

One benefit to the use of the E1000 Electropuls indenter in the model was its accuracy; it can be set to very precise rates and forces and maintain them over a long period of time. However, its disadvantages were that this machine is not present in most labs and will need specific training for access, and the change in location also may create issues with keeping the experiment sterile.

As the reduction in culturable bacteria was similar across chewing mechanisms, it gives the potential for both to be used. When conducting small experiments with low replicates, the faster manual, modified Wessel chewing process could be used. However, when completing experiments which use many discs, such as a CDC bioreactor run, the indenter could prove more reliable due to the removal of human error.

2.6 Conclusions and Further work

This chapter investigated the development of a model which allows biofilms to grow in the CDC bioreactor flow system before being exposed to the mechanical forces of chewing gums via the E1000 Electropuls indenter or the modified Wessel method.

This model was validated and compared to the Zurich-based model for growth. It was observed that the CDC bioreactor has a lower number of culturable bacteria within the biofilms. However, as discussed earlier there were some potential technical issues with getting complete bacterial counts from the discs, this should be investigated further. The biofilm proved to be more resilient to mechanical shear forces when grown in the reactor. This is likely to be connected to the biofilm being thicker in the CDC reactor than in the Zurich-based model. It is hypothesized that this is due to the flow of media the biofilms are exposed to in the CDC bioreactor; this shear flow will encourage thicker EPS, creating a more robust biofilm. This thicker, more resilient biofilm will represent the biofilms in the oral cavity well due to the shear forces exposed to the oral biofilms throughout the day.

This model modified the bacterial exposure to chewing gum from the Wessel method (Wessel *et al.*, 2015); it characterised the bacterial removal from chewing gum when in a biofilm state. It was observed that when using the manual modified Wessel method, there was no significant difference from the E1000 indenter, mechanical method. The development of the mechanical chewing method to a standard which correlates to the modified Wessel method was an important step for testing chewing gums on biofilms. It allows for high reproducibility with less human error whilst utilising basic lab equipment, which is more available and appropriate for an academic setting than the ERWEKA machine. This means that when testing small quantities of biofilms, the modified Wessel method could be used, but when processing large quantities of biofilms, the E1000 indenter would be more appropriate.

Further characterisation of this model could be carried out to investigate how the two growing systems affect EPS production. This could be done using a matrix stain, such as SYPRO Ruby, to stain the proteins present in the EPS. When paired with SYTO 9, the EPS and viable cells could be imaged together to determine biofilm density. Another validation step would be to use an *ex vivo* inoculum to determine if the model would bias bacterial growth; this could be tested using 16S sequencing before and after the use of the model.

Chapter 3:

Assessing the efficacy of antimicrobial agents against *Streptococcus mutans and* exploring the impact of incorporating them into chewing gum formulations.

"All the people living in our United Netherlands are not as many as the living animals that I carry in my mouth this very day."

Antonie van Leeuwenhoek

Chapter 3 Assessing the efficacy of antimicrobial agents against *Streptococcus mutans* and exploring the impact of incorporating them into chewing gum formulations.

3.1 Introduction

There is a long history of the use of chewing gums for pleasure and to improve oral health, which dates back to the Aztecs (Mathews, 2009). More recently, chewing gums have had active compounds added to increase oral health benefits. The first medicated chewing gum (MCG) patent was filed in 1869 (Jacobsen, Christrup and Jensen, 2004), with the first MCG being launched in 1924 before the concept gained traction in the 1970s with the invention of nicotine chewing gums (Russell, Raw and Jarvis, 1980; Woodford and Lesko, 1981). The use of chewing gums as an antimicrobial release system into the oral cavity could be used to help mitigate oral diseases, which are experienced by 56% of people during their lifetime (Abbafati *et al.*, 2020).

Oral diseases are linked to oral microbiome dysbiosis, which occurs due to a shift in the oral biofilm's community. Within the oral cavity, biofilms can form in all seven areas of the mouth (Dewhirst *et al.*, 2010), with biofilms in the gingival-sulcus region being some of the hardest to remove as they are the hardest to access. In addition to the protection from the oral gingiva, biofilms provide added layers of protection to the bacteria. This protection comes in the form of reduced diffusion of antimicrobials through the EPS, the development of persister/viable but non-culturable cells, and protection from shear forces in their environment (Tseng *et al.*, 2013; Wood, Knabel and Kwan, 2013).

There are eight potential antimicrobials of note in relation to this chapter. These were chosen based on their potential as oral antimicrobials from literature searches. The first was chlorhexidine, a well-established oral antimicrobial commonly used in medicated mouthwashes (Chye *et al.*, 2019). Secondly, there are the essential oils, including; cinnamaldehyde (Firmino *et al.*, 2018; He *et al.*, 2019), methyl salicylate (Vlachojannis *et al.*, 2015), menthol and eucalyptol. These are also used in mouthwashes as oral antimicrobials. Essential oils have been tested against key oral bacteria, for example, cinnamaldehyde against *S. mutans* (He *et al.*, 2019), a key oral coloniser known for its potential to cause oral disease.

Other promising oral antimicrobials evaluated were LL37, cranberry extract and propolis (Górniak, Bartoszewski and Króliczewski, 2019). These have also been tested against key oral bacteria, and bactericidal effects were shown. LL37 is an antimicrobial peptide produced in the oral cavity by neutrophils (Dale *et al.*, 2006). Cranberry extract and propolis are natural products with antimicrobial properties. Yamanaka et al. observed cranberry extract's ability to reduce *Streptococcus spp*. adsorbing to the tooth's surface (A. Yamanaka, 2004), whilst propolis has been characterised by Wojtyczka et al. as having a bactericidal effect against *Staphylococcus aureus* (Wojtyczka *et al.*, 2013).

With the known effects of these antimicrobials against oral microbes, their incorporation into chewing gums could be very promising. The addition of the mechanical action of chewing could increase their efficacy. Chewing gum alone was seen to remove oral bacteria (Takahashi *et al.*, 2003). If the gum is disrupting the oral biofilms, antimicrobials may be able to penetrate deeper and have a greater effect. Characterising the effects of antimicrobial chewing gums on *S. mutans* biofilms has the potential to provide novel insights into biofilm management in the oral cavity.

3.2 Chapter aims

The first aim of this chapter was to investigate the minimum bactericidal concentrations of eight potential antimicrobials against planktonic *Streptococcus mutans.* These antimicrobials and concentrations were chosen based on a literature search. Based on the measured effectiveness of the antimicrobials, a few select antimicrobials were then taken forward for further evaluation.

The second aim of this work was to develop and optimise a chewing gum formulation which incorporates antimicrobials, followed by the determination of the release of these antimicrobials rates from the chewing gums during mechanical chewing.

The final aim of this chapter was to characterise the combination of antimicrobials and the mechanical chewing effects on biofilm culturable counts and architecture and to determine if this effect was additive or synergistic.

3.3 Methods

3.3.1 Bacterial strains and culturing

The bacterial strains used were *Streptococcus mutans* UA159 from the ATCC and genetically modified (GM) *Streptococcus mutans* UA159 that includes plasmid pVA8912, a erythromycin resistance plasmid (Banasb, 2015). This GM strain was provided by Professor Nick Jakubovics and Dr Halah Ahmed from the University of Newcastle. *S. mutans* was chosen due to the important role it plays in the early colonisation of the oral cavity and oral diseases such as dental caries.

S. mutans was cultured on BHI agar (Merck) overnight at 37°C in a CO_2 incubator to confirm purity. A single colony was then selected and inoculated into 10 ml of BHI broth (Merck) and incubated overnight at 37°C in a CO_2 incubator to create an overnight culture. To create glycerol stocks, 750 µl of this overnight culture was added to 750 µl of 50% glycerol (ThermoFisher) in a cryovial and stored at -80°C.

For the *S. mutans* pVA8912, BHI agar with 10 ng/ml of erythromycin (Sigma-Aldrich) was used to create a streak plate, which was incubated overnight at 37°C in a CO_2 incubator. A single colony was selected and inoculated into 10 ml of BHI broth with 10 ng/ml of erythromycin, then incubated overnight at 37°C in a CO_2 incubator. Glycerol stocks were made from this overnight culture and stored in the -80°C freezer.

Planktonic growth rates were characterised by adding 20 µl of planktonic suspension to 10 ml of BHI broth in a Falcon tube. Absorbance readings were collected every 60 mins up to 12 hours at 601 nm using a Jenway 6300 spectrophotometer, and CFUs were plated out. This was done by completing a 10-fold serial dilution, seven times sequentially in PBS (Thermo Fisher), by adding 20 µl to 180 µl of PBS to produce final concentrations ranging from 100% to 0.00001%. These were spot plated onto a BHI agar plate with three spots of 10 µl and incubated overnight at 37°C in a CO_2 incubator. This was repeated at 24 hours. The number of colonies present at each dilution were counted. The minimum number of colonies able to be calculated was 99, based on the experimental design and calculation.

3.3.2 *Streptococcus mutans* exposure to antimicrobials

3.3.2.1 *S. mutans* UA159 planktonic MIC/MBC data

Minimum inhibitory concentration (MIC) testing of eight antimicrobials was conducted; these antimicrobials were chosen based either on their appearance in other oral healthcare products or

their potential oral antimicrobial properties. The MIC of each compound was found using a literature search; concentrations and references are in Table 1. Dilution of the antimicrobials was carried out in PBS. Eucalyptol (Sigma-Aldrich) and menthol (Merck) were dissolved in 5% dimethyl sulfoxide (DMSO) (Thermo Fisher) and PBS to improve their solubility. Due to the refractivity of the essential oils, the use of the spectrometer to detect growth inhibition was unusable. This caused a change to MBC experiments rather than MIC experiments.

In a 96-well plate, 100 µl of *S. mutans* UA159 overnight culture was added to 100 µl of the antimicrobial solution. The antimicrobial stock solutions were made up at twice the intended maximum concentration to account for this dilution. These were then serially diluted 2-fold, with PBS, seven times to achieve a range of final concentrations of 16X to 1/8th of the literature-reported MIC (see Table 1). A positive bacterial control was also included, with 100 µl of overnight culture added to 100 µl of PBS and a negative control of 200 µl of PBS. The 96-well plate was incubated in a 37°C 5% CO_2 incubator overnight. To assess the effect of the antimicrobials, each concentration was then serially diluted so a countable number of bacteria were present and spot plated. Each antimicrobial under test had two technical repeats and three biological repeats conducted.

Table 1: A summary of the eight antimicrobials used in this screening, whether they were dissolved in 5% dimethyl sulfoxide, their minimum inhibitory controls from the literature and which concentrations of each antimicrobial was used.

Antimicrobial compound	Dimethyl sulfoxide (5%) added	Minimum inhibitory concentration from the literature	Concentrations used in experiment
LL37 (Merck)	No	37 µg/l (Leszczyńska *et al.*, 2013)	4.625, 9.25, 18.5, 37, 74, 148, 296 and 592 µg/l
Methyl Salicylate (Merck)	No	3% (Xu, Ou and Wu, 2018)	0.375, 0.75, 1.5, 3, 6, 12, 24 and 48%
Eucalyptol (Sigma-Aldrich)	Yes	250 µg/ml (Martínez-Pabón and Ortega-Cuadros, 2020)	31.25, 62.5, 125, 250, 500, 1000, 2000 and 4000 µg/ml
Cinnamaldehyde (Merck)	No	1000 µg/ml (He *et al.*, 2019)	125, 250, 500, 1000, 2000, 4000, 8000 and 16000 µg/ml
Menthol (Merck)	Yes	100 µg/ml (Martínez-Pabón and Ortega-Cuadros, 2020)	12.5, 25, 50, 100, 200, 400, 800 and 1600 µg/ml
Cranberry extract (Holland & Barrett)	No	25 µg/ml (Singhal *et al.*, 2020)	3.125, 6.25, 12.5, 25, 50, 100, 200 and 400 µg/ml
Chlorhexidine (Merck)	No	0.24% (Löe and Rindom Schiøtt, 1970)	0.03, 0.06, 0.12, 0.24, 0.48, 0.96, 1.92 and 3.84%
Propolis (Bee&You)	No	0.035 µg/ml (Przybyłek and Karpiński, 2019)	0.035 and 0.07 µg/ml

3.3.2.2 Introduction of plasmid variant *Streptococcus mutans*

S. mutans pVA8912 was used to replace the wild-type strain in order to prevent the continuation of recurrent and sporadic contamination by *Bacillales* (Appendix A pg.130). By using an erythromycin resistant *S. mutans,* erythromycin could be added to the BHI media and BHI agar preventing extraneous contamination without affecting the growth of *S. mutans*. *S. mutans* pVA8912 growth curves were recorded to determine the growth rate compared to wild type *S. mutans.*

3.3.2.3 *Streptococcus mutans* UA159 and *Streptococcus mutans* pVA8912 planktonic 15 minutes exposure to four of the most promising antimicrobials

A streak plate and overnight culture were grown as previously stated for the *S. mutans* wild type and plasmid variant. In a 96-well plate, 100 µl of *S. mutans* overnight culture was added to 100 µl of antimicrobial solution. For 15-minutes the 96-well plate was incubated in a 37°C CO_2 incubator. This was then serially diluted, 10-fold seven times in PBS, by adding 20 µl of the antimicrobial-overnight mix to 180 µl of PBS and spot plated as before. Using this spot plating method, the minimum amount of detectable viable bacteria that can be detected was 99 CFU per ml.

3.3.2.4 *Streptococcus mutans* pVA8912 biofilms 15 minutes exposure to four key antimicrobials

A streak plate and overnight culture was grown as previously stated for the *S. mutans* wild type and plasmid variant. Sterile hydroxyapatite coupons were added to a 12-well plate, with 1 ml of overnight culture and 1 ml of $1/5^{th}$ strength BHI broth made up with PBS. This was placed in the 37°C, CO_2 incubator for 72 hours. Every 24 hours 1 ml of broth was removed from the well, and 1 ml of fresh $1/5^{th}$ BHI broth was added gently down the side of the well. After 72 hours, all the broth was removed from each well and replaced with 1X the MIC for each of the four key antimicrobials. The biofilms were exposed for 15 minutes, the antimicrobial was removed, and the discs washed in 2 ml of PBS.

The discs were then either scraped using a polyethylene cell scraper, serially diluted 10-fold and then spot plated or they were LIVE/DEAD stained for confocal imaging. For the cell scraping, the wash was removed, and 1 ml of fresh PBS was added directly onto the biofilm. The cell scraper was then scraped over the disc for 30 seconds. The PBS was then pipetted up and down onto the disc three times before 200 µl was removed and added to a 96-well plate. This was then serially diluted and spot plated as previously mentioned.

For the confocal laser scanning microscopy, a LIVE/DEAD stain was prepared by creating a stock of 2 µl of propidium iodide (Thermo Fisher) and 2 µl of SYTO9 (Thermo Fisher) per 1 ml of PBS. The discs were then exposed to the stain for 10 minutes before washing with PBS. The discs were allowed to partially dry before 40 µl of Mowiol hard set resin (Sigma-Aldrich) was added to the top of the disc; they were then heated at 45°C for 30 minutes before a coverslip was placed on top.

Samples were imaged using an inverted Leica SP8 confocal laser scanning microscope. A 63X oil immersion lens was used with Argon (488 nm) and DPSS (561 nm) lasers, scanning sequentially at 1 µm intervals. Three images were taken for each disc, in identical places on each sample to avoid bias. Initially to get to set the correct focus a FITC filter was used on the first sample site. Images were obtained using the LAS X software, and quantitative image analysis was completed on the IMARIS software.

3.3.3 Optimisation of the chewing gum formulation

3.3.3.1 Reduced ingredient chewing gum formulation

A reduced-ingredient chewing gum formulation was trialled to reduce the potential for overlap of the peaks of the active ingredients with those of the other gum ingredients when analysing the release of actives from the gum by UV-vis spectrometry. The exact ingredients list, quantities and brands are proprietary information and therefore have not been included. A z-blade mixer (L-range Winkworth) was preheated to 80°C whilst the ingredients were weighed out, a single bulk sweetener and a gum base. No flavourings were added to the control chewing gum. The sweetener was added to the z-blade mixer, and mixing started. The gum base was melted to a liquid consistency in a microwave. This melted gum base was then added to sweetener in the z-blade mixer and mixed forward for 3 minutes and in reverse for 1 minute following a Mondelēz standard operating protocol (SOP). This mixture was left to cool to 60°C before being moved onto a work surface prepared with greaseproof paper with a dusting of bulk sweetener. This mix was then rolled out to a $1/8^{th}$ of an inch thick and cut into chewing gum pellet shapes.

3.3.3.2 Optimisation steps for the reduced ingredient chewing gum formulation

To improve gum cohesion several improvements were tested. The reduced ingredient chewing gum formulation was repeated with the addition of an emulsifier. The emulsifier was added to the melted chewing gum base and stirred together before being added to the sweetener in the z-blade mixer and mixed. The effects of the z-blade temperature on chewing gum consistency were

tested with a range of 60°C to 95°C. The length of each mixing stage was tested at two further time points: 5 minutes forward and 1 minute in reverse and 10 minutes forward and 1 minute in reverse.

3.3.3.3 Full chewing gum formulation

The z-blade mixer was preheated to 80°C whilst the ingredients were weighed out; three bulk sweeteners, two high-intensity sweeteners, a gum base, and an emulsifier. The three bulk sweeteners were sieved and added to the z-blade mixer to combine them. The gum base was melted to a liquid consistency in a microwave, and once melted the emulsifier was added and combined. This liquid mix was then added to the sweetener combination in the z-blade mixer and mixed forward for 3 minutes and in reverse for 1 minute. The intense sweeteners were finally added to the z-blade mixer and mixed forward for 3 minutes and in reverse for 1 minute. This mixture was left to cool to 60°C before being moved onto a work surface prepared with greaseproof paper dusted with bulk sweetener. This mix was then rolled out to the set 1/8th inch width and cut into chewing gum pellet shapes.

3.3.3.4 Variations of the full chewing gum formulation

In replacement to the flavourings, antimicrobials were added to the chewing gums in a range of concentrations depending on the minimum inhibitory concentration (MIC) of each antimicrobial against *S. mutans*. These MIC values were chosen from a literature search. Chlorhexidine (0.096% and 0.192%, which equates to 0.4X and 0.8X the MIC), cinnamaldehyde (125 µg/ml, 250 µg/ml, 500 µg/ml and 1000 µg/ml, equating to 0.125X, 0.25X, 0.5X, and 1X the MIC), methyl salicylate (3.9 mg/ml which equates to 0.13X MIC), and propolis (0.035 µg/ml, and 0.07 µg/ml which equates to 1X and 2X the MIC).

3.3.3.5 Mechanical mastication of the chewing gum

Each of the formulated chewing gums were mechanically chewed for 5, 10 or 15 minutes. The machine used imitates the mastication process; however, this method cannot be disclosed as it is a Mondelez proprietary method and is confidential. These time points were chosen as 15 minutes was used as the standard length in chewing gum trials (Woodford and Lesko, 1981; Duizer, Bloom and Findlay, 1996; Simons *et al.*, 2001), and the 5 and 10-minute time points allow for the investigation of how the antimicrobials are released over this chew time. For each chew time, a piece of chewing gum was added to a compartment of the machine in PBS. It was then chewed for the required amount of time before being removed and filtered using an 8 µm ashless filter paper (Whatman). The chewing gum bolus was stored at -20°C in a freezer, and the chewing gum

diffusate was stored in a Falcon tube in a 4°C fridge. This was repeated twice for each chewing gum concentration.

3.3.4 Investigating compound release over time and the different chewing gum antimicrobial concentrations

3.3.4.1 Collecting calibration curve data for each antimicrobial

For each of the four antimicrobials selected for more in-depth evaluation (chlorhexidine, cinnamaldehyde, methyl salicylate, and propolis), a range of concentrations were made up in pure ethanol (ThermoFisher). After blanking the UV-vis spectrometer using pure ethanol, whole spectral scans were completed on each sample at a mid-range concentration to determine their spectral peak. These were found to be 260 nm for chlorhexidine, 285 nm for cinnamaldehyde, 300 nm for methyl salicylate, and 326 nm for propolis. A range of concentrations of each compound was then tested at their spectral peak to determine their linear regressions; see Table 2 for concentrations and Appendix D (138) for example graphs.

Table 2: Range of concentrations of each antimicrobial used in the chewing gums for the UV-vis spectrometry to determine the linear regression of each antimicrobial.

Compound	Range of concentrations used
Chlorhexidine (% v/v)	0.00002, 0.0002, 0.00025, 0.0005, 0.001, 0.002, 0.0025 and 0.005
Cinnamaldehyde (µg/ml)	10, 50, 100, 500 and 1000
Methyl Salicylate (% v/v)	0.000015, 0.00003, 0.00015, 0.0003, 0.0015 and 0.003
Propolis (ng/ml)	0.0175, 0.175, 0.35,1.75, 3.5 and 35

3.3.4.2 Investigating compound release using the UV-vis spectrometer

The UV-vis spectrometer was blanked using the appropriate time related control chewing gum fluid, then 2 ml of each of the diffusates was added to a quartz cuvette and tested at their

respective wavelength. If the signal was too strong (above 2 absorbance units), dilutions of 1 in 2, 1 in 10 and 1 in 100 were made using ethanol. For these, the UV-vis spectrometer was re-blanked using an appropriate time related control chewing gum which had been diluted in ethanol to the same dilution. The absorbances collected were then compared to the calibration curve absorbance data, and the amount released from each concentration was calculated.

3.3.5 Determining the effects of the chewing gum diffusate on *S. mutans*

3.3.5.1 *S. mutans* planktonic exposure to chewing gum diffusates

Due to the refractivity of the compounds interfering with the optical density reads, MBC experiments were carried out in place of MIC experiments. This experiment was carried out using the same protocol as the MBC experiments in 3.3.2.1, with the exception of chewing gum diffusates which were used in replacement of the antimicrobials. The gum diffusates used were from the chlorhexidine 0.8X MIC, methyl salicylate 0.13X MIC, the propolis 1X MIC, cinnamaldehyde 1/8X MIC, 1/4X MIC, 1/2X MIC, and 1X MIC chewing gums.

3.3.5.2 *S. mutans* biofilm 15-minute exposure to chewing gum diffusates

This experiment was carried out using the same protocol as the biofilm exposure experiments in 3.3.2.4, with the exception that chewing gum diffusates were used in replacement of the antimicrobials. The gum diffusates used were from the chlorhexidine 0.8X MIC, cinnamaldehyde 1X MIC, and propolis 1X MIC chewing gums.

3.3.6 Exploring the effects of antimicrobials on *S. mutans* biofilm when in the presence of chewing

This experiment was carried out using the same protocol as the biofilm exposure experiments in 3.3.2.4, with the addition of mechanical chewing of the biofilms whilst exposed to the antimicrobial. The antimicrobial used was cinnamaldehyde at two concentrations, 1000 μg/ml and 4000 μg/ml.

3.3.6.1 Statistical analysis

The statistical tests used in this chapter are stated in the figure legends of each figure. The two most common statistical tests used were an unpaired T-test and ANOVA. For the characterisation of UV-vis spectrometry data of the compound concentrations, a linear regression was used. GraphPad Prism 9 was used to run the statistical analyses. In all statistics, a significance level of

$P<0.05$ was used, with all values above this being classified as non-significant. For the bacterial counts, the raw data was log-transformed before a mean with SD was performed; this normalises the data.

3.4 Results

3.4.1 *Streptococcus mutans* exposure to antimicrobials

3.4.1.1 The exposure of planktonic *S. mutans* to eight antimicrobials

Figure 17 shows results for how *S. mutans* culturability is affected by the presence of antimicrobials at a range of concentrations around their MIC according to a literature search. Planktonic *S. mutans* cultures were exposed to eight antimicrobials and compared to their controls, shown in Figure 17. The recorded values for the control planktonic growth were $2x10^9$ culturable *S. mutans* bacteria and $5x10^8$ culturable *S. mutans* bacteria when exposed to 5% DMSO. The two antimicrobials dissolved in DMSO, eucalyptol and menthol, had no significant difference from their control. Cranberry extract and LL37 also showed no significant difference from the control at any concentration. Only the four highest concentrations of chlorhexidine were significantly different from the control. Methyl salicylate was significantly different from the control, only at the highest concentration. All concentrations of cinnamaldehyde and both concentrations of propolis were significantly different from the control. The doubling of the antimicrobial concentration was expected to create a downward trend in bacterial culturability; however, this was not seen with LL37, eucalyptol, menthol, or cranberry extract. Chlorhexidine and methyl salicylate did show this trend. For cinnamaldehyde exposure, a very high bactericidal effect was observed; it was only at a concentration of 125 μg/ml that bacteria were present. Propolis was only tested at two concentrations; both concentrations showed a significant reduction in the culturability of *S. mutans* planktonic bacteria. For each of the antimicrobials, an ANOVA was completed (see Appendix B pg. 134).

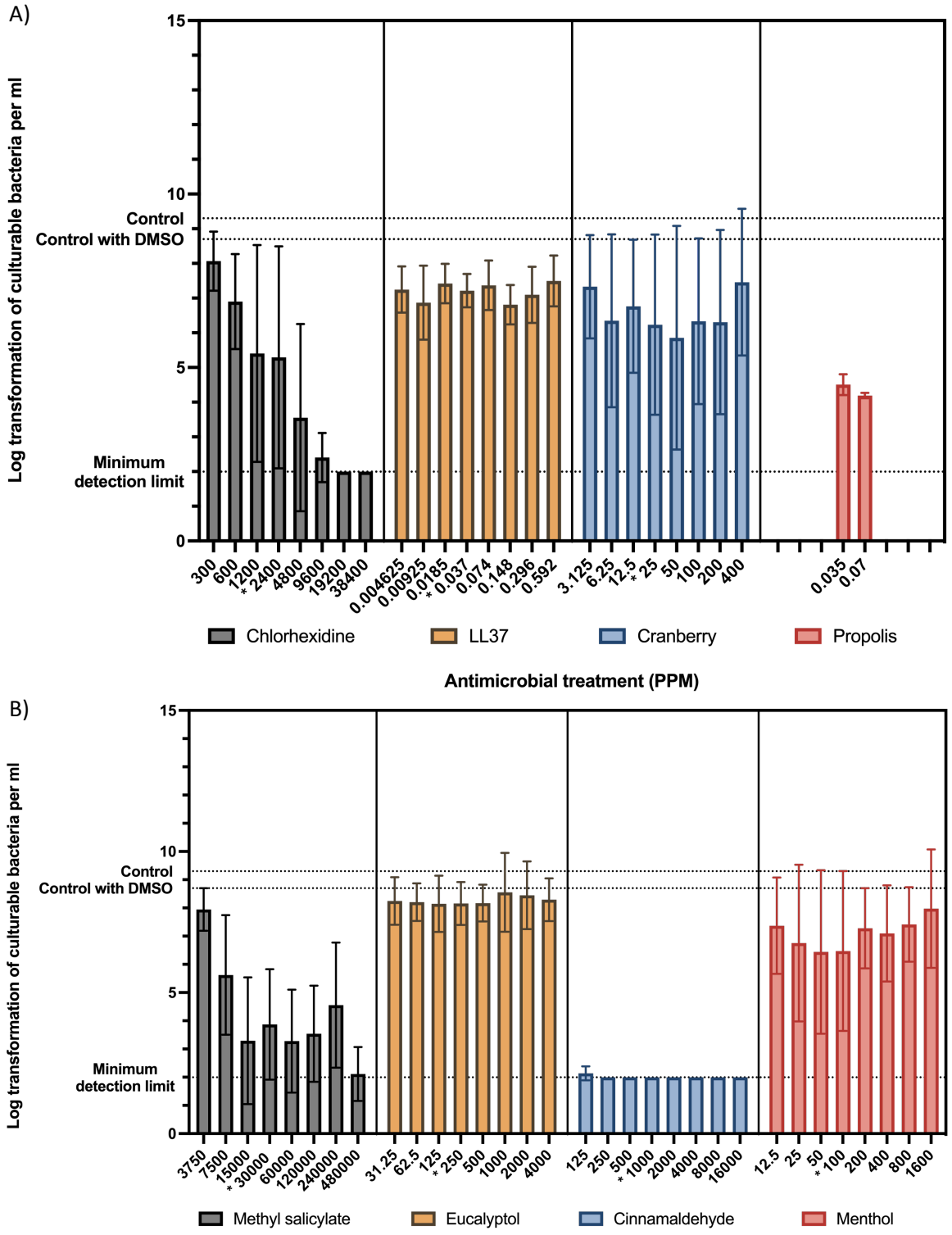


Figure 17: The log transformation of the number of culturable Streptococcus mutans planktonic bacteria after exposure to eight antimicrobials for 20 hours. Each antimicrobial has eight concentrations ranging from 1/8th of the minimum inhibitory concentration to 16X the minimum inhibitory concentration. 1X the minimum inhibitory concentration is shown by an * under the ppm of each antimicrobial. These minimum inhibitory concentrations were based on literature data. The upper dotted line shows the control with no antimicrobial present, and the middle-dotted line shows the control with no antimicrobial but with dimethyl sulfoxide, as eucalyptol and menthol had 5% dimethyl sulfoxide added to help keep them in solution. The line at 1.99 indicates the lowest detection limit for each biological repeat. Mean ± 1 standard deviation, n=3. A) shows the non-essential oil antimicrobials, B) shows the essential oil antimicrobials.

3.4.1.2 Comparative growth curve for *S. mutans* wild type and plasmid pVA8912 variant

Due to continued contamination a plasmid variant of *S. mutans* was used so erythromycin could be added to the media and agar. Comparisons of *S. mutans* wildtype and *S. mutans* pVA8912 growth curves were completed to determine if the plasmid affects bacterial growth or antimicrobial efficacy, as shown in Figure 18. The plasmid variant *S. mutans* had a lower initial number of culturable bacteria starting at $1X10^5$, compared to *S. mutans* wild type $2X10^6$. They followed the same exponential phase increase until they both entered the stationary phase at around $1X10^9$ culturable bacteria.

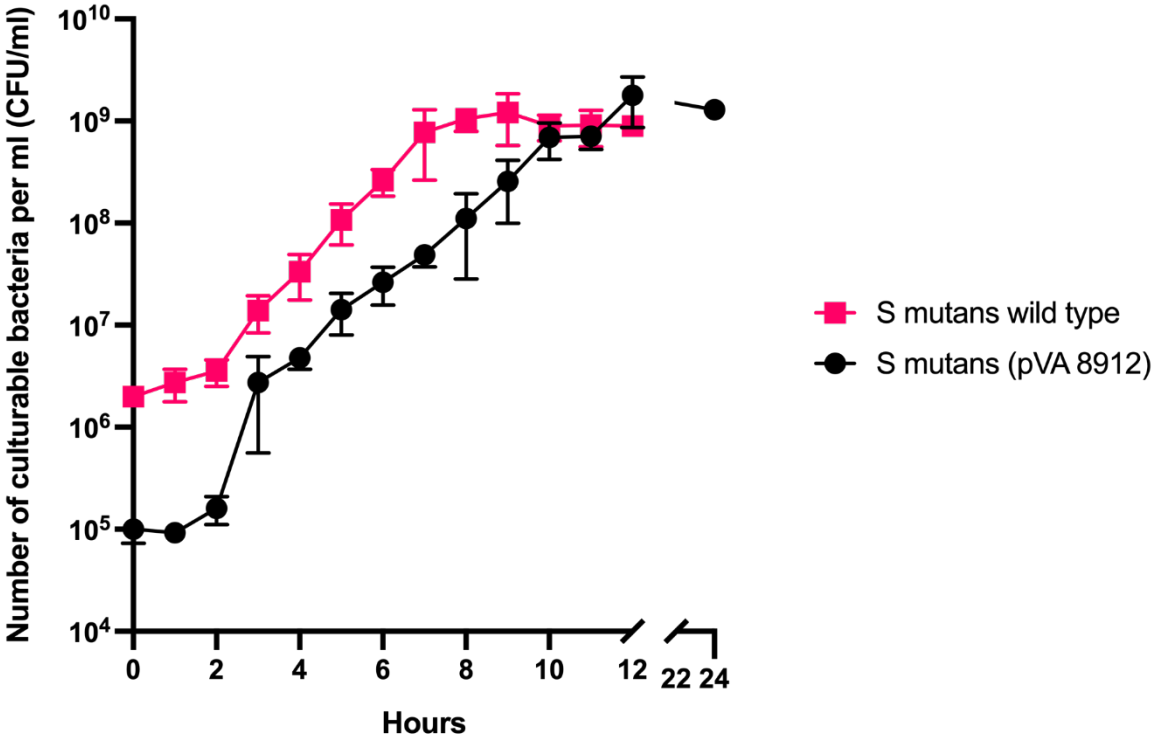


Figure 18: Growth curves for both *Streptococcus mutans* wild-type and *Streptococcus mutans pVA8912. Streptococcus mutans* wild type (pink) was grown in brain heart infusion, with the *Streptococcus mutans* pVA8912 (black) grown in brain heart infusion with 10 ng/ml erythromycin. This shows the growth of both species over the first 12 hours, with an extra time point for the plasmid variant at 24 hours. The mean ± 1 standard deviation, and n=3, is shown.

3.4.1.3 Comparison of the response of wild-type and pVA8912 *S. mutans* planktonic cells to antimicrobial exposure

After testing the growth rate of *S. mutans* pVA8912 against *S. mutans* WT, the promising antimicrobials from Figure 17 were then tested on both planktonic *S. mutans* pVA8912 and planktonic *S. mutans* WT to determine any differences in efficacy. Unpaired t-tests were conducted to investigate the differences between the wild-type and plasmid variant *S. mutans* to selected antimicrobial compounds. Each antimicrobial exposure was compared to its plasmid mutant counterpart. Figure 19 shows both *S. mutans* strains had a similar quantity of culturable bacteria and effect from chlorhexidine when in planktonic form, with no significant difference (P=0.07 and P=0.84, respectively). Cinnamaldehyde reduced the number of culturable bacteria for both the wild-type and plasmid variant below the detectable limit. Methyl salicylate had a slightly reduced effect on the plasmid variant compared to the wild type; however, this difference was not significant (P=0.06). The effect of propolis on the plasmid variant of *S. mutans* was significantly less effective (P=0.01) than when compared to propolis' effect on the wild-type *S. mutans* there was a 3-log increase in bacterial counts.

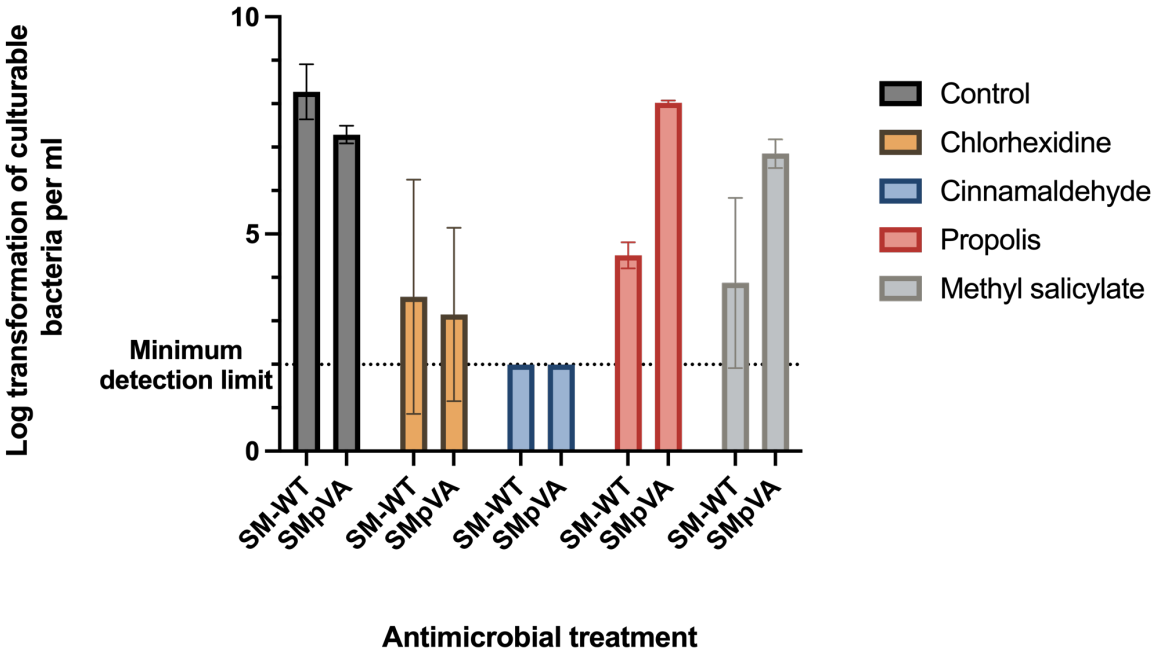


Figure 19: Comparison of wild type and SMpVA mutant *Streptococcus mutans* when exposed to antimicrobials. The log transformation of the number of culturable *Streptococcus mutans* planktonic bacteria, both wild type and pVA8912 variant, after exposure to four key antimicrobials for 15 minutes. *Streptococcus mutans* wild type was grown in brain heart infusion, with the *Streptococcus mutans* pVA8912 grown in brain heart infusion with 10 ng/ml erythromycin. The antimicrobials were exposed at 1X the minimum inhibitory concentration; chlorhexidine 0.24%, cinnamaldehyde 1000 μg/ml, methyl salicylate 3%, and propolis 0.035 μg/ml. The line at 1.99 indicates the lowest detection limit for each biological repeat. For both bacterial variants, a mean ± 1 standard deviation and n=3 is shown.

3.4.1.4 The exposure of 3-day biofilm *S. mutans* to three key antimicrobials for 15 minutes

The next step to investigating the differences between the wildtype and variant was to test the antimicrobials against them as biofilms. Initial experiments of the antimicrobials on biofilm *S. mutans* wildtype were then compared to the *S. mutans* pVA8912 and plotted in Figure 20. The wildtype *S. mutans* has a significantly lower control compared to the *S. mutans* pVA8912 plasmid variant ($P<0.05$). At these concentrations of antimicrobials, there was not a significant effect on bacterial culturable counts; however, a similar trend was observed across both *S. mutans* strains.

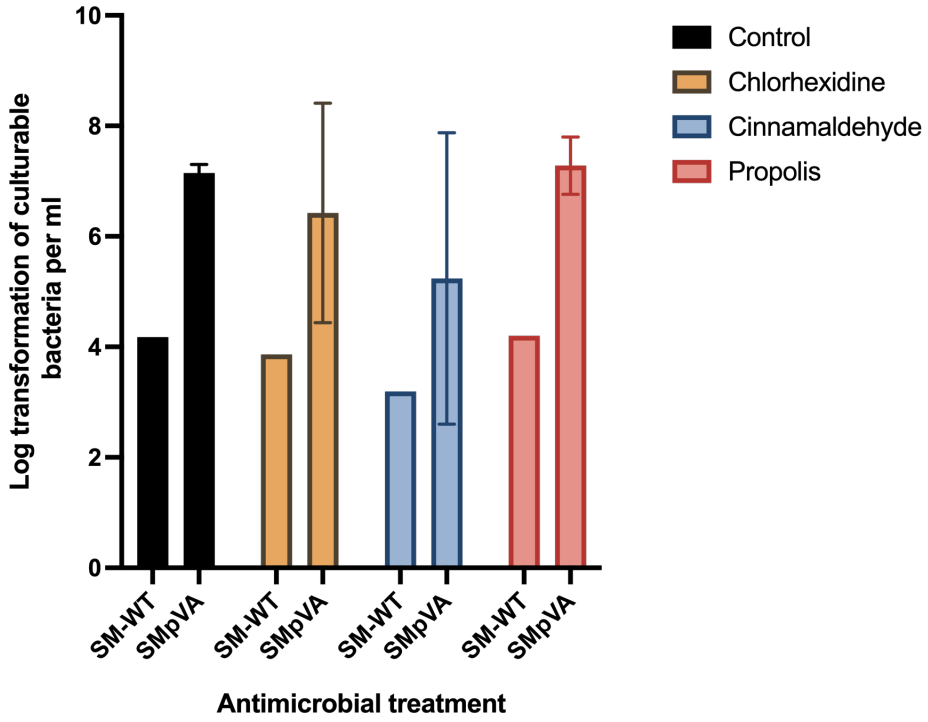


Figure 20: The log-transformed number of culturable, 3-day biofilm *Streptococcus mutans* bacteria after exposure to four key antimicrobials for 15 minutes. *Streptococcus mutans* wild type was grown in brain heart infusion, with the *Streptococcus mutans* pVA8912 grown in brain heart infusion with 10 ng/ml erythromycin. The antimicrobials were exposed at 1X the minimum inhibitory concentration; chlorhexidine 0.24%, cinnamaldehyde 1000 μg/ml, and propolis 0.035 μg/ml. For *S. mutans* wild type, n=1 was obtained (due to contamination affecting results collection); for *S. mutans* pVA8912, n=3 replicates were obtained, and a mean ± 1 standard deviation was calculated.

In Figure 21, four representative, confocal images of 3-day *S. mutans* pVA8912 plasmid variant biofilms exposed to antimicrobials for 15 minutes are shown. This will show what effects the antimicrobials are having on the biofilm architecture, to be compared with the CFU counts. The control biofilm shows an 80 μM thick biofilm with a large number of live and dead bacteria, a slightly thinner biofilm was seen after chlorhexidine exposure at 64 μM. However, when the biofilms were exposed to cinnamaldehyde and propolis, a large reduction in biofilm thickness was observed, dropping to 20 μM and 32 μM, respectively, with a large decrease in live bacteria

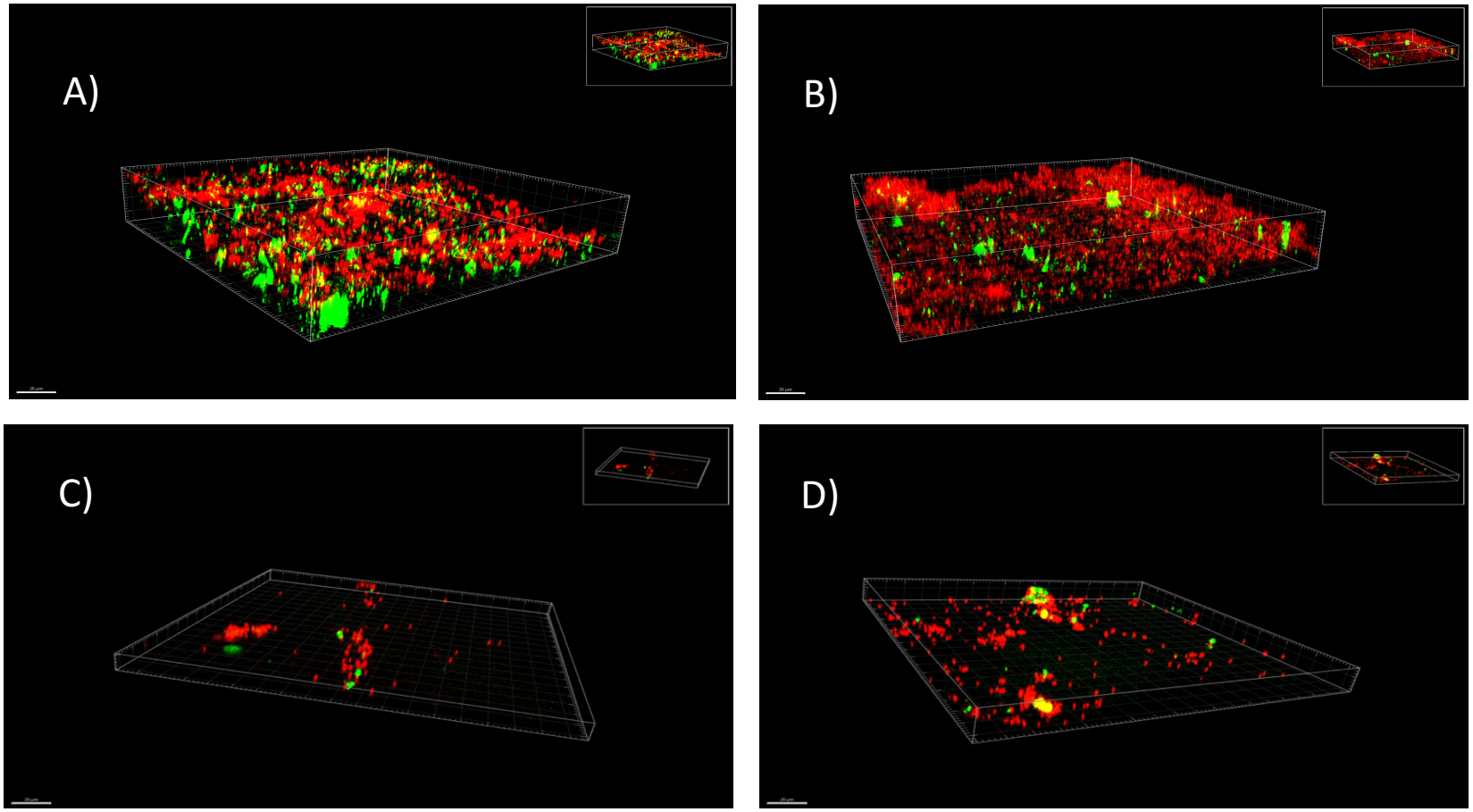


Figure 21: Laser scanning confocal microscopy images of a 3-day *Streptococcus mutans* pVA8912 plasmid variant, exposed to antimicrobials for 15 minutes. The biofilms were then stained with LIVE/DEAD stain and set in Mowiol hard set resin. LIVE/DEAD stain contains SYTO9 a green stain for live bacteria and propidium iodide a red stain for dead bacteria. A) phosphate buffered saline control biofilm, B) chlorhexidine exposed biofilm, C) cinnamaldehyde exposed biofilm, and D) propolis exposed biofilm.

3.4.1.5 Development and optimisation of the chewing gum formulation

The first step in making the antimicrobial chewing gums was to create a control chewing gum using a reduced ingredient formulation. This reduced formulation was used to minimise the interaction of the peaks from the chewing gum ingredients and the active compounds when using the UV-vis spectrometer. The original reduced ingredient formulation came together in the z-blade mixer; however, upon cooling, it became very crumbly and was unable to be rolled out and cut into shape, as seen in Figure 22A and Figure 23A. This made it hard to portion correctly, and with this texture, when added to the chewing machine for chewing, it broke apart further and partially dissolved into the PBS.

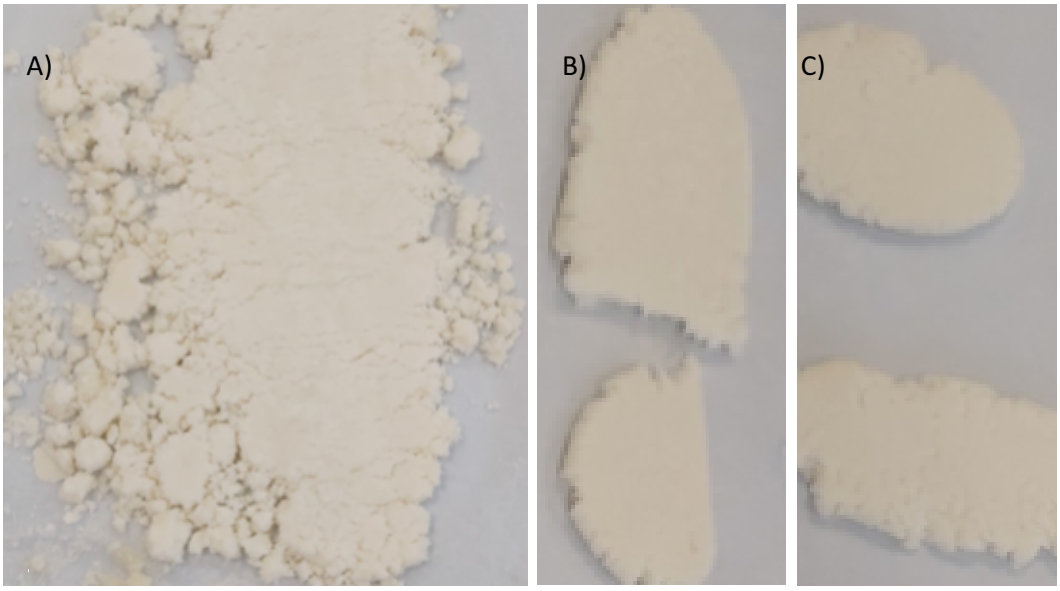


Figure 22: A) Photograph of the reduced ingredient chewing gum with an emulsifier added, mixed at 80°C. B) as 22A but mixed at 85°C. c) as 22A but mixed at 95°C.

With the addition of an emulsifier, a small improvement was seen in the cohesion of the chewing gum; however, it was still too dry and crumbly to be used. Increasing the mixing temperature from 80°C to 95°C showed further improvement; however, when mechanically chewed, it also broke apart and dissolved into the PBS. These improvements are shown in Figure 22B and Figure 22C. To remedy this, a complete formulation was used.

In the expanded formulation with more sweeteners included, better cohesion of the ingredients was observed. The chewing gum was now able to be rolled out and cut, as seen in Figure 23B. The size of each piece differs slightly; however, they all weigh 2 grams +/- 0.2 grams. When added to the chewing machine and chewed, this formulation stayed together and formed a solid chewing

gum bolus. This allowed the progression to the creation of antimicrobial-containing chewing gums.

Figure 23: A) Reduced ingredient chewing gum showing the gum to be dry and crumbly, B) full ingredient chewing gum cut into 2-gram pieces.

When formulating the antimicrobial chewing gums, there was a limit to the quantity of antimicrobial/flavour replacement which could be added. The maximum amount of methyl salicylate that could be incorporated into this gum only equated to 0.13X MIC since higher amounts prevented the gum from forming a solid structure.

3.4.2 Determining antimicrobial release rates against chew time and chewing gum concentrations

To determine the concentration of antimicrobials being released, a UV-vis spectrometer was used to correlate UV-vis absorbance with antimicrobial concentration using linear regression analysis. The absorbance data of a range of antimicrobial concentrations, taken at their spectral peak, was used to create calibration curves for each antimicrobial. The linear regression graphs for chlorhexidine, cinnamaldehyde and propolis are displayed in Appendix D (138). Methyl salicylate could not be completed due to the refractivity of the compound in the UV-vis spectrometer.

3.4.2.1 Investigating compound release using the UV-vis spectrometer

Using the linear regression curves, each antimicrobial was then tested to determine the antimicrobial release concentration. At each cinnamaldehyde concentration, the percentage of antimicrobial released increased with chew time (Figure 24). This was also true for propolis and chlorhexidine; however, due to the very small percentage of compound released, it was much

harder to measure; the maximum percentage of propolis released was 1% from the 2X MIC chewing gum at 15-minute chew time. The highest release percentage was from the 1/2X MIC cinnamaldehyde gum, at 19.4%. The lowest was from the chlorhexidine gum, where there was no detectable chlorhexidine release at 0.4X MIC at any time point.

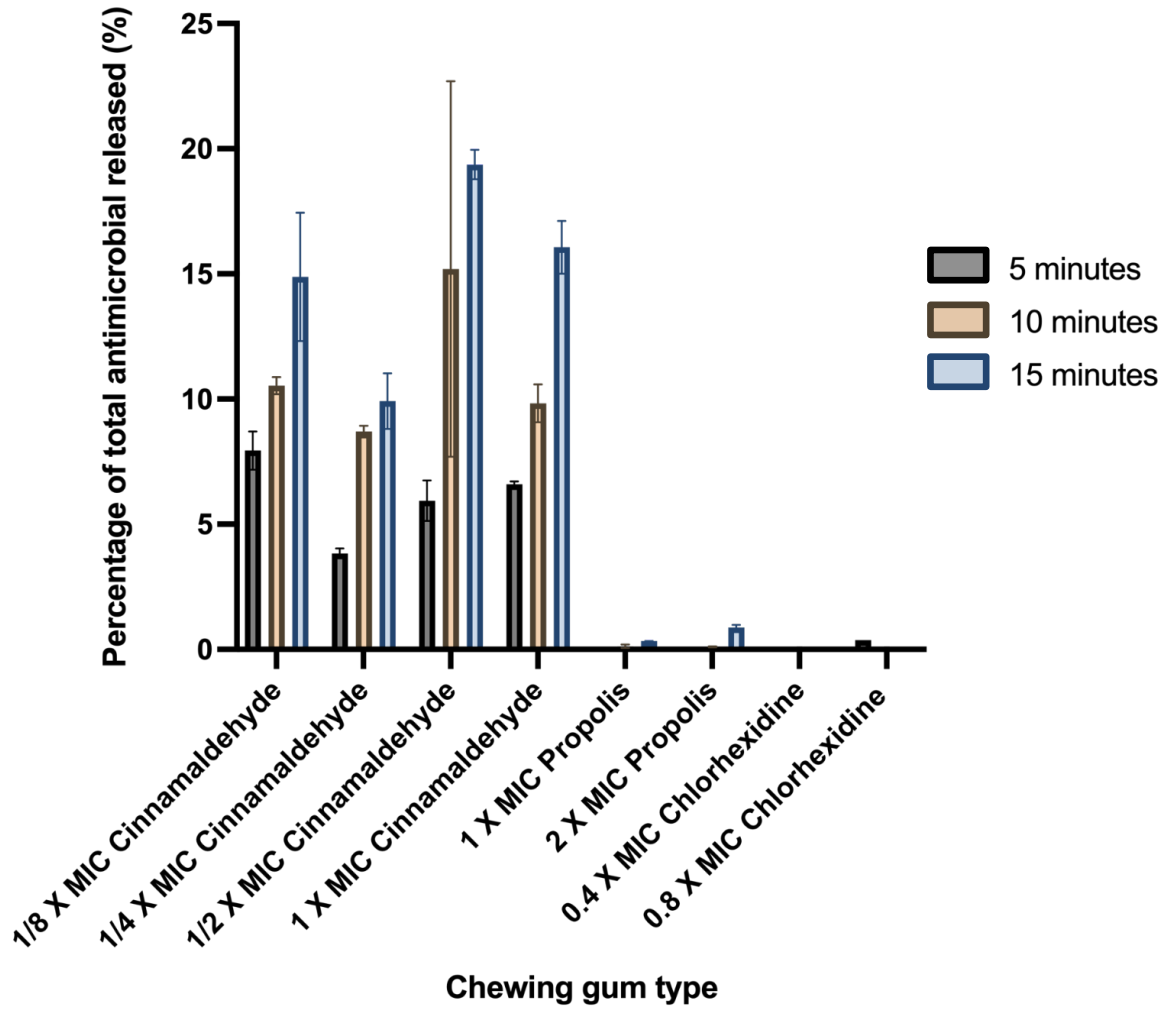


Figure 24: The percentage of an antimicrobial released from a 2-gram piece of chewing gum over three chewing times; 5 (grey), 10 (yellow), and 15 minutes (blue), shown as a percentage of the total quantity of antimicrobial added to the chewing gum piece. The chewing gum was chewed in phosphate buffered saline and the percentage was investigated using UV-vis spectroscopy and linear regression graphs. Mean ± 1 standard deviation, n=3.

3.4.2.2 Characterising *S. mutans* response to antimicrobial chewing gum diffusate

The chewing gum diffusate from the mechanical chewing was then tested on planktonic *S. mutans* to see if exposing the bacteria to these representative levels of antimicrobial from the chewed gum would be effective. Figure 25 shows that at five, ten and fifteen minutes, there was no significant difference between the control chewing gum and the bacterial control (shown as a dashed line). This was also true for the methyl salicylate and chlorhexidine chewing gums. With

cinnamaldehyde and propolis, the five- and ten-minute diffusates show no significant difference from the control chewing gum. However, at the fifteen-minute chew time, no culturable bacteria were detected, so the minimum detectable limit was plotted; this was significantly different from the bacterial control (p=0.0001). The bactericidal effect seen in these experiments was limited by the spot-plating method. In this system, the minimum number of culturable bacteria was 99 per ml. This means that if fewer than 99 bacteria are culturable in the well, then it may be missed and labelled as a full bactericidal effect; the minimum number of culturable bacteria was plotted on the graphs.

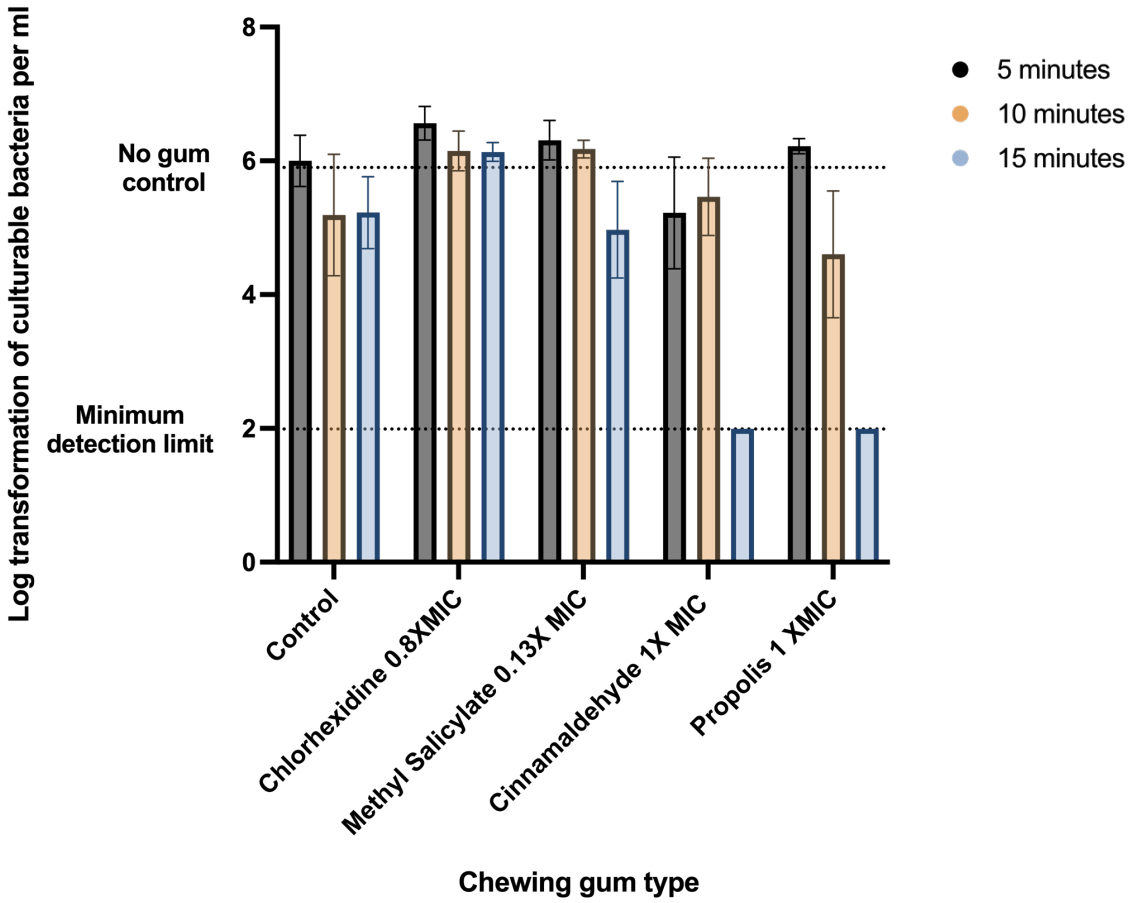


Figure 25: The log transformation of the number of culturable *Streptococcus mutans* after being exposed to four types of antimicrobial chewing gum diffusate overnight; chlorhexidine, methyl salicylate, cinnamaldehyde and propolis, compared to a control chewing gum diffusate with no antimicrobial presence. Each diffusate has three bars, one for each length of time the chewing gum was mechanically chewed for, five minutes in grey, ten minutes in yellow and fifteen in blue. The control number of culturable bacteria which were exposed to chewing gum diffusate is shown as a dashed line. The line at 1.99 indicates the lowest detection limit for each biological repeat. Mean and ± 1 standard deviation of the mean, n=3.

Due to the reduction in culturable bacteria in Figure 25 from exposure to cinnamaldehyde chewing gum diffusate further testing was completed for different cinnamaldehyde concentrations (see Figure 26). Cinnamaldehyde at 1/8 X and 1/4 X the MIC, all three chewing lengths, do not show a reduction in number from the control bacteria, shown as the upper dashed

line. This was also true for the five-minute chew time for 1/2 X the MIC chewing gum diffusate and the five- and ten-minute chew times for the 1 X the MIC chewing gum diffusate. However, at the ten-minute chew time for the 1/2 X MIC, a significant decrease was observed (P=0.03). At the 15-minute chew time for both 1/2 and 1 X the MIC chewing gum diffusates, there are no culturable bacteria counted; therefore, the minimum detection limit was plotted. This indicates the level released by these two chewing gums after fifteen minutes of chewing has a strong antimicrobial effect against planktonic *S. mutans*.

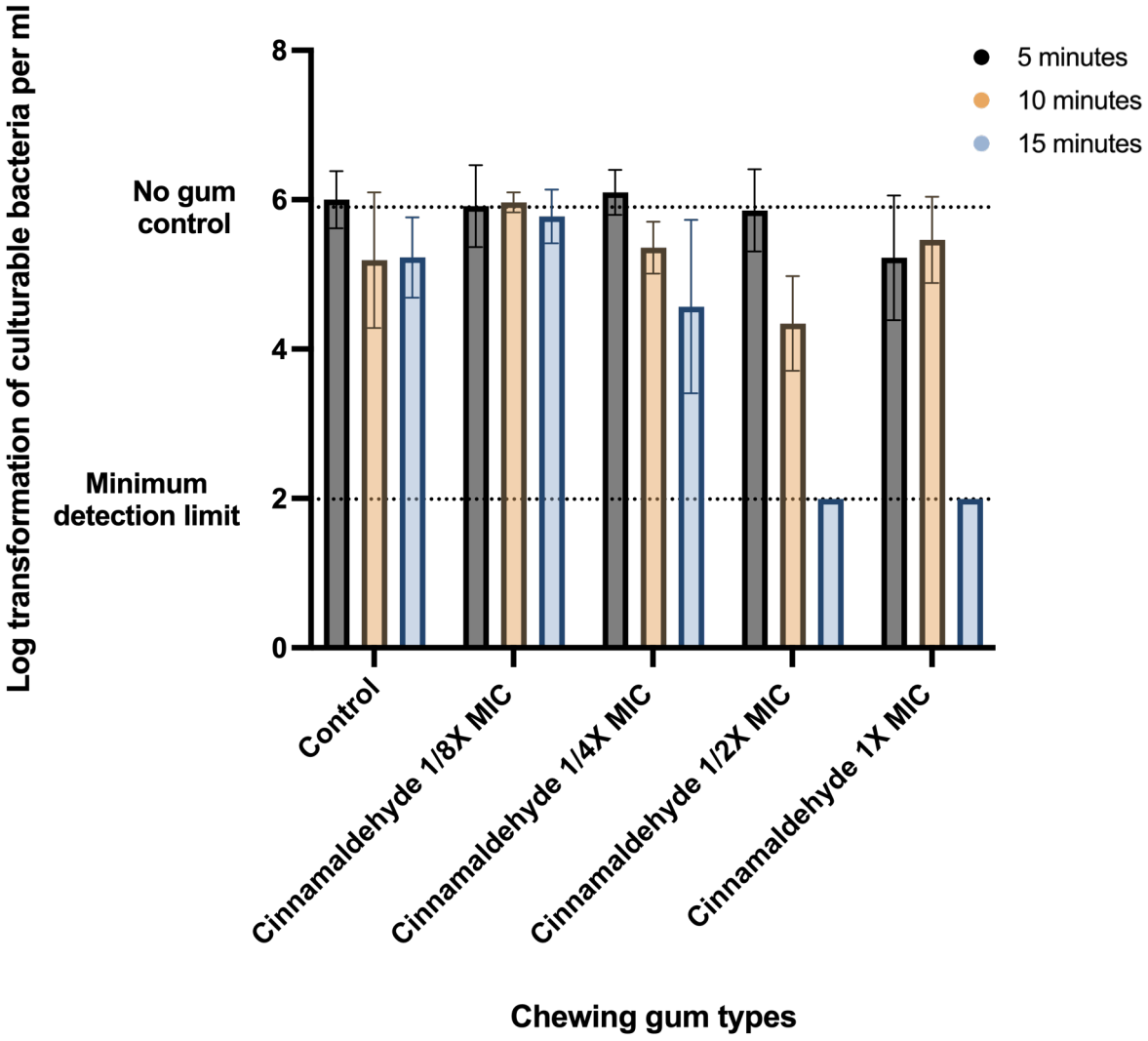


Figure 26: Log transformation of the number of culturable planktonic *Streptococcus mutans* bacteria after being exposed to four concentrations of antimicrobial chewing gum diffusate overnight; cinnamaldehyde at 1/8X minimum inhibitory concentration, 1/4X minimum inhibitory concentration, 1/2X minimum inhibitory concentration and 1X the minimum inhibitory concentration. Each diffusate has three bars, one for each length of time the chewing gum was mechanically chewed for, five minutes in grey, ten minutes in yellow and fifteen in blue. The number of culturable bacteria exposed to phosphate buffered saline with no chewing gum diffusate, is shown as a dashed line. The line at 1.99 indicates the lowest detection limit for each biological repeat. Mean ± 1 standard deviation, n=3.

3.4.2.3 Characterising biofilm *S. mutans* response to antimicrobial chewing gum diffusate

The next step was to assess how these gum diffusates affected *S. mutans* pVA8912 when in the biofilm state. When assessing the CFU counts of 3-day *S. mutans* biofilms after exposure to the antimicrobial chewing gum diffusates, Figure 28 shows that none of the chewing gum diffusates influenced the number of culturable bacteria. An ANOVA test was carried out, comparing each of the chewing gum diffusates with the control, which shows that none of them were significantly different. Chlorhexidine P=0.98, cinnamaldehyde P=0.86, propolis P=0.93 and methyl salicylate P=0.98.

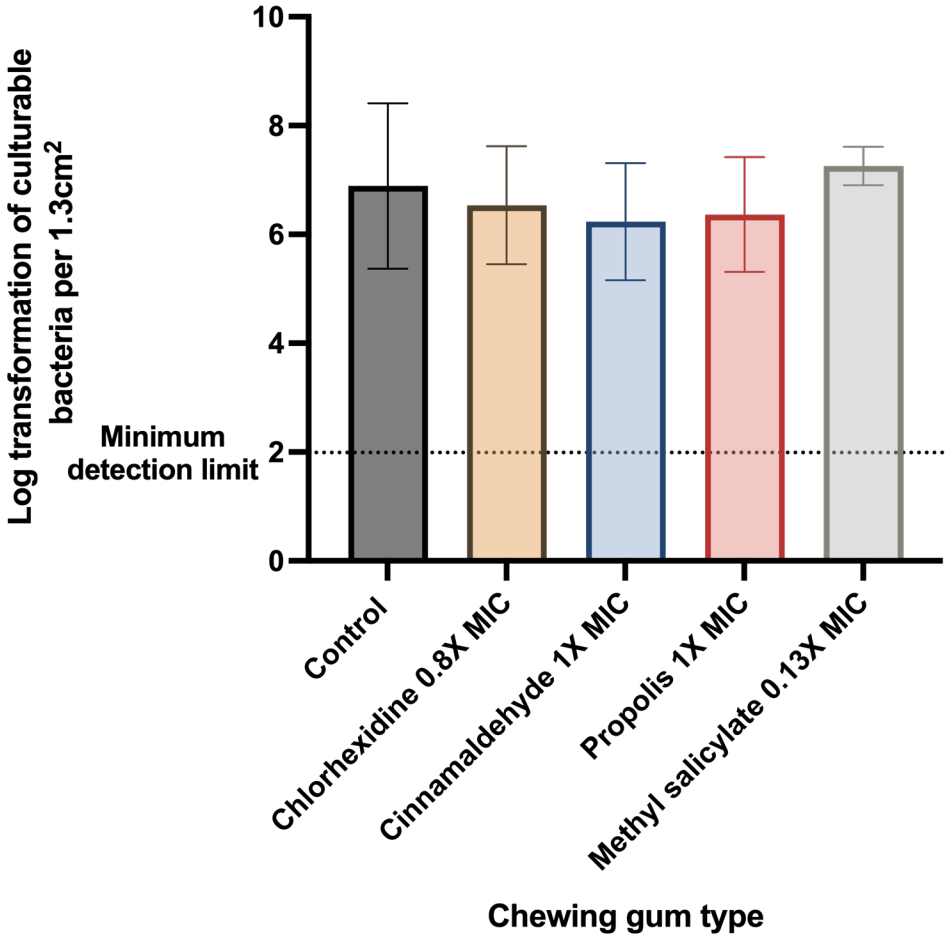


Figure 27:Bar chart of the log-transformed number of culturable biofilm *Streptococcus mutans* after being exposed to four types of antimicrobial chewing gum diffusates for 15 minutes; a control gum with no antimicrobials added, chlorhexidine at 0.8x the minimum inhibitory concentration (MIC), cinnamaldehyde at 1x the MIC, propolis at 1x the MIC, and methyl salicylate at 0.13X the MIC. The line at 1.99 indicates the lowest detection limit for each biological repeat. Mean ± 1 standard deviation, n=3.

3.4.3 Combining the mechanical removal of chewing gum with the presence of cinnamaldehyde on 3-day *S. mutans* biofilms

To characterise the effects of chewing and antimicrobials on 3-day biofilms; two concentrations of cinnamaldehyde were tested; for the potential of synergism. To determine if there is synergism, the two treatments would have interacted and created a further mechanism of action or increased efficacy. Figure 28 shows each of the concentrations of cinnamaldehyde alone, exposed for 15 minutes, and with chewing. The addition of chewing does significantly reduce the culturable count compared to cinnamaldehyde exposure alone.

When comparing the chewed control to the chewed in the presence of cinnamaldehyde, the 1X MIC causes no further reduction to the culturable count (P=0.61). However, when increasing the cinnamaldehyde to 4X MIC concentration with chewing, a very significant reduction to the culturable count is observed (P=0.0002).

The potential of synergism was characterised by investigating the raw bacterial counts shown in Table 3. For both treatments, where the biofilms were exposed to 1X the MIC and 4X the MIC of cinnamaldehyde, the culturable CFUs were counted. This was repeated for the chewed biofilm treatment. The combined number of killed bacteria for cinnamaldehyde and chewed were added together, and this combined effect was larger than when the biofilm was exposed to cinnamaldehyde whilst chewing. This means that the effect was not synergistic and was suggested as additive (Noel, Keevil and Wilks, 2021).

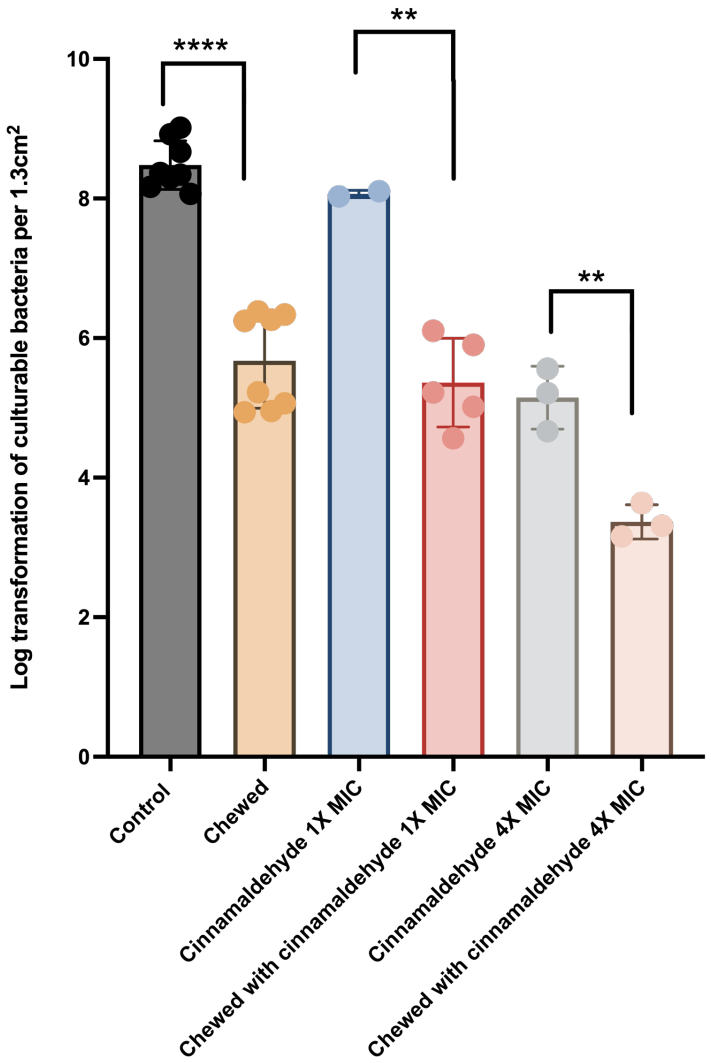


Figure 28: Bar chart showing the log transformation of culturable 3-day biofilm *Streptococcus mutans pVA8912* after being exposed to two concentrations of cinnamaldehyde (1000 μg/ml and 4000 μg/ml), mechanical chewing and mechanical chewing with cinnamaldehyde (1000 μg/ml and 4000 μg/ml). Each treatment was 15 minutes, followed by a phosphate-buffered saline wash step. A mean ± 1 standard deviation, and n=8 for control and chewed, n=3 for cinnamaldehyde 1X minimum inhibitory concentration (MIC), cinnamaldehyde 4X MIC and chewed with cinnamaldehyde 4X MIC, and n=5 for chewed with cinnamaldehyde 1X MIC. *= p=0.05, **= p=0.01, ***= p=0.001, ****= p=0.0001.

Table 3: This table shows the average control value minus the average treatment value. Also shown is the calculated chewed minus control and calculated cinnamaldehyde minus control combined.

Treatment	Average control minus average treatment (Number of culturable bacteria per 1.3cm^2)
Chewed	4 X10^7
Cinnamaldehyde (1X MIC)	3 X10^7
Chewed with cinnamaldehyde present (1X MIC)	4 X10^7
Chewed + cinnamaldehyde (1X MIC) - separately	7 X10^7
Cinnamaldehyde (4X MIC)	4 X10^7
Chewed with cinnamaldehyde present (4X MIC)	4 X10^7
Chewed + cinnamaldehyde (4X MIC) - separately	9 X10^7

3.5 Discussion

Over half the global population (56%) experience an oral health disease during their lifetime, with some experiencing the follow-on life-changing, systemic diseases which can occur as a result of the systemic inflammation caused by oral disease. Finding novel biofilm control mechanisms and combining these with commonly used consumer practices (such as chewing gum) may help reduce oral biofilms and improve oral health. This chapter focused on evaluating a pool of oral antimicrobials for their potential to improve oral health. The results indicate cinnamaldehyde and propolis could be incorporated into chewing gums as a helpful additive to oral hygiene regimes.

3.5.1 The exposure of *S. mutans* to antimicrobials

The investigation of the literature search MIC data showed that the reported MIC was different from our characterised MBC. With the same media and strain being used, the inhibition did not occur at the expected concentration for all the tested compounds. In Figure 17, chlorhexidine, cinnamaldehyde, methyl salicylate, and propolis showed strong antimicrobial properties against *S. mutans* planktonic bacteria. Cinnamaldehyde proved much more effective than expected from the literature search, with a bactericidal effect at 250 µg/ml rather than a MIC at 1000 µg/ml (He *et al.*, 2019). Methyl salicylate was reported as having a MIC value of 3% (Xu, Ou and Wu, 2018), and Figure 17 observed an bactericidal effect at 1.5%. In Figure 17, propolis was observed to have an inhibitory effect on bacterial culturability; this supports the previously described MIC value of 17 µg/ml (Przybyłek and Karpiński, 2019). The cranberry extract showed a slight but non-significant decrease at all concentrations. The literature observes cranberry extract to have an anti-adsorption effect on *S. mutans* (Yoo, Murata and Duarte, 2011), which would only occur when in the biofilm formation stages.

Eucalyptol, menthol, cranberry extract and LL37 are not significantly different from their controls, as shown in Appendix B (pg 134). These compounds did not exhibit a concentration dependant effect; to see this, a much wider set of concentrations would need to be tested to observe a concentration which has an effect and to reach the MBC value. Furthermore, the observed 2-log reduction in bacterial counts may not be sufficient for practical applications, particularly against bacterial biofilms. Based on this testing, only three of the antimicrobials were taken forward for further testing; cinnamaldehyde, methyl salicylate, and propolis. Chlorhexidine was also used in experiments as a reference, as it is a well-known -used agent commonly used in commercial oral care products.

The introduction of *S. mutans* pVA8912 was essential to reduce experimental disruption due to contamination, as described in Appendix A (pg 130). This plasmid variant allowed for experiments to be carried out in the presence of erythromycin. Figure 18characterised the growth of both wild-type *S. mutans* and the plasmid variant; whilst the variant had a lower starting culturable count, the final count after 12 hours was similar. Both strains followed a similar growth pattern, entering the exponential phase at similar times.

Only cinnamaldehyde continued to show a promising bactericidal effect against planktonic *S. mutans* wild type and pVA8912 with a short exposure time (Figure 19). The concentrations, however, were not effective against the 3-day biofilm growth of *S. mutans*, which was attributed to the added protection afforded to bacteria in a biofilm state. When comparing the effects of antimicrobials between the two strains, chlorhexidine shows a similar decrease in planktonic culturable counts. However, the effect of cinnamaldehyde was slightly reduced in pVA8912 compared to the wild type, with methyl salicylate and propolis causing no reduction in the culturable counts. This indicates methyl salicylate and propolis are less effective against the pVA8912 strain than the wild type.

3.5.2 Chewing gum development and characterisation

Initially, a reduced ingredient chewing gum formulation was used. The aim of this was to reduce the potential interference of the ingredients in the spectrometry readings, with more ingredients creating more peaks. Some of the ingredients may also affect the bacterial CFU experiments, such as xylitol and its known effects on *S. mutans* (Nayak, Nayak and Khandelwal, 2014). However, due to the change in texture and stability of this gum, the required experiments could not be performed, so the full ingredient formulation was used.

The reduced ingredient formulation created a very crumbly chewing gum. This was because it used sorbitol as the single sweetener; sorbitol is known to form crystals in food matrices. This crystallisation affects the texture of the chewing gum, with a high crystalline presence leading to short, crumbly chewing gum (DeJong and Hartel, 2020). This can be remedied by using additional sweeteners. The addition of other sweeteners, such as mannitol, increases the time it takes to start crystal formation (DeJong and Hartel, 2021), whilst xylitol is known to reduce the crystallisation of sugars in food matrices (Young and O'Sullivan, 2011). This explains why the reduced ingredient chewing gum had a crumbly texture, and the addition of other sweeteners remedied this.

When conducting the UV-vis spectrometer experiments with the full ingredient chewing gum, no overlap between the chewing gum and antimicrobial peaks was observed. There was also no effect on the bacterial viability in the experiments conducted on control chewing gums (without active ingredients) – so, in fact, using the full formulation did not cause any challenges with the analysis.

Once a viable chewing gum formulation was developed, antimicrobials were added; however, a full 1X MIC concentration was not possible for methyl salicylate. Methyl salicylate has a MIC of 3% in the saliva, which equates to 154 grams of methyl salicylate per 500-gram batch of chewing gum, which would prevent the chewing gum from forming a solid gum pellet. Based on this, a 0.13X MIC chewing gum was created. Due to the refractivity of methyl salicylate, the linear regression curve could not be determined; this led to no release concentrations being determined. The diffusate from the 0.13X MIC methyl salicylate chewing gum had no effect on the planktonic *S. mutans*. This highlights the importance of using antimicrobials with high efficacy for their incorporation into chewing gums to be viable.

Cinnamaldehyde had the greatest release of the antimicrobial chewing gums, with 19% of the total antimicrobial being released after 15 minutes of mechanical chewing. Each of the cinnamaldehyde chewing gums, at each time point had some antimicrobial release, varying from 4-19%. Figure 24 showed that as chew time increased, so did the percentage of antimicrobial released, with 15 minutes consistently having the highest release. Cinnamaldehyde was hypothesized to have released well due to it being a small molecule (MW=132) and having no charge (Information, 2023); these may both increase release from the chewing gum.

When propolis gum was chewed for 15 minutes, a release of 1% of the added propolis in the 2X MIC chewing gum was calculated from the linear regressions at 326 nm. When chewed for 5 minutes, no propolis release was detected. After 10 minutes of chewing, only 0.1% was released on average.

Data shown in Figure 24 suggests no chlorhexidine was released from the 0.4 X MIC chewing gum over the 5, 10 or 15-minute chewing period; however, at the 5-minute chew time for the 0.8 X MIC chewing gum, a small release occurred; this was not seen at longer chewing times. This reduction of chlorhexidine release over chewing time was the opposite of what was observed in the other antimicrobials and indicated there might be an error in the reading. It could indicate that the amount "released" was within the error range of the spectrometer and that it was calculated incorrectly, or it could be indicating that the chlorhexidine was binding to something in the PBS and precipitating out of the solution. This was hypothesised as the chewing gums are

chewed in PBS, any chlorhexidine released may have interacted with the NaCl present in the PBS and precipitated out of solution (Zeng *et al.*, 2009). When chewing the 10 and 15-minute chewing gums, they are exposed to 30 ml of PBS rather than 20 ml; this may make a difference in the precipitation of the chlorhexidine.

If the indicated released quantity was an error in the UV-vis reading, the low release of chlorhexidine could be due to its large molecular weight, MW=897, when bound with gluconate salts (PubChem, 2023). The second hypothesis was the gluconate salts the chlorhexidine is bound with may be binding strongly to the chewing gum molecules and reducing release. This was indicated as CHewX®, a commercially available chewing gum containing chlorhexidine in the acetate form, was able to inhibit plaque growth (Imfeld, 2006).

Chlorhexidine gluconate was the chlorhexidine product used in medicated mouthwashes and had been demonstrated to be effective against oral bacteria (Brookes, Zo¨e L.S. ,Bescos, 2020). Chlorhexidine diacetate was a chlorhexidine product in acetate salt form, commonly used in disinfectants and anti-fouling biocides (Guo *et al.*, 2015; Chug *et al.*, 2022). Both chlorhexidine mixtures have strong antimicrobial properties. This led me to use chlorhexidine gluconate in the antimicrobial chewing gums. However, upon further literature searches, previous chlorhexidine incorporating chewing gums, like CHewX®, used chlorhexidine diacetate (Imfeld, 2006). Due to the ability of CHewX® to remove oral bacteria, the use of the acetate salt form of chlorhexidine rather than the gluconate salt form may be required for an effective release and delivery of potential antimicrobial efficacy.

When exposing planktonic *S. mutans* to chewing gum diffusates, cinnamaldehyde gums at 1/2X MIC and 1X MIC, 15 minutes chew time, caused a complete bactericidal effect. Cinnamaldehyde was shown in (Figure 17) to have a higher efficacy than was published in the literature. This explains the bactericidal effect observed with only a 19% release rate; this correlated to 1/5X the MIC. In Figure 17, at 1/4X the MIC, there was a bactericidal effect, but at 1/8X the MIC, there were culturable bacteria present at a level lower than the control, indicating a bactericidal effect. The bactericidal effect from both the cinnamaldehyde chewing gum and propolis chewing gum was only observed at the 15-minute chewing time.

When planktonic *S. mutans* was exposed to the propolis diffusate of 1X MIC, with a 15-minute chew, it was able to cause a complete bactericidal effect on planktonic *S. mutans* (Figure 25). This is a stronger effect than when bacterial cells were exposed to propolis alone. This may indicate a product of the chewing gum is interacting with the propolis and improving its bactericidal activity.

The bactericidal capability of the propolis diffusate indicated that although very little propolis was released from the gum, the fraction of propolis which was released had a strong antimicrobial effect. Propolis is made up of many components; this level of bactericidal effect indicates the fraction of propolis being released has a strong antimicrobial effect or is interacting with the chewing gum diffusate. High-performance liquid chromatography (HPLC) has previously been used to investigate which fraction of propolis was responsible for its antimicrobial properties; it was suggested polyphenolic compounds were the cause of the antimicrobial effects (Veloz, Alvear and Salazar, 2019).

González et al investigated four of the key polyphenolic compounds in propolis; caffeic acid, galangin, chrysin and pinocembrin. These were observed to have bactericidal effects against *Helicobacter pylori* (Romero *et al.*, 2019). Their individual spectral peaks were 300 nm-350 nm, 261 nm and 351 nm, 270 nm, and 290 nm, respectively(Kim, Kim and Jung, 2008; Belay, 2012; Park and Park, 2017; Yang *et al.*, 2018). However, the collective spectrum for propolis was measured at 326; therefore, if these compounds are being released when chewed, they may not be picked up by the UV-vis spectrometry. Whilst the fractions with the spectra at 326 nm may not be released from the gum, this limitation could be improved using a full spectral scan rather than a single point scan.

The bactericidal effect occurring only at the 15-minute chew time for both cinnamaldehyde and propolis gums highlights the importance of chew time on antimicrobial release and, by extension the efficacy of the chewing gum. Due to the low release rate of chlorhexidine from the chewing gum, Figure 25 shows that the diffusate was not effective against the planktonic *S. mutans.* Overall, the lack of effect from the other chewing gum chew times and concentrations indicated that the reduced release of the antimicrobials inhibits their antimicrobial efficacy, as the released concentrations were below the MIC value.

When the chewing gum diffusates were exposed to 3-day *S. mutans* biofilms, there was also no significant reduction in bacterial culturability, as seen in Figure 28. This was expected due to the lack of reduction in the culturable counts when exposed to 1X the MIC of each antimicrobial on 3-day *S. mutans* biofilms (Figure 20); therefore, as less than 1X MIC was released from the chewing gums, no effect was expected. This reduction in antimicrobial efficacy was hypothesized to be due to the added protection bacteria receive from entering a biofilm state. The bacteria in a biofilm have reduced contact with the antimicrobials due to the EPS creating diffusion gradients in conjunction with some cells entering a more dormant state. The mechanical disruption of this biofilm state, by shearing forces from tooth brushing or chewing gum, could increase the efficacy

of antimicrobials by reducing EPS protection and allowing the antimicrobials to penetrate deeper into the biofilms.

In Figure 29, 3-day *S. mutans* biofilms were exposed to mechanical chewing, cinnamaldehyde, and mechanical chewing and cinnamaldehyde, a significant reduction was seen in culturability for the mechanically chewed biofilm; however, no significant reduction was seen for the cinnamaldehyde (1X MIC) exposed biofilm. When combined, the result was significantly different from the control and from the chewed treatment; this means that the presence of cinnamaldehyde had a significant effect when used in combination with chewing. This was repeated with a higher concentration of cinnamaldehyde (4000 μg/ml); this confirmed the effect was additive, not synergistic. Whilst chewing gum allows the cinnamaldehyde to work more effectively, the reduction in culturability seen in Table 3 shows it to be additive.

For synergism testing, experiments usually characterise absolute bactericidal effect not a reduction in culturability. In addition to this, synergism experiments often compare two chemical treatments, whereas here, chemical and mechanical treatments were investigated. Only a single concentration and chewing parameters were tested; for conclusive data for additive or synergism, more concentrations were needed. Usually, for a synergistic effect to occur, the two chemicals interact and produce a third mechanism of action, which differs from the original two. In this experiment, the mechanical removal allowed the chemical treatment to be more effective; however, this was not synergistic.

3.6 Conclusions and Further work

Eight antimicrobials with the potential to benefit oral health were tested. Even with the same media and strain type, the MBCs observed in Figure 17 differ from those reported in the literature. Cinnamaldehyde and propolis were observed to be highly effective against planktonic *S. mutans*, even at low concentrations. Whilst methyl salicylate and chlorhexidine caused a concentration-dependent decrease in culturable bacteria, indicating that the range of tested concentrations were close to the MIC concentration. Whereas the tested concentrations for eucalyptol, menthol, cranberry and LL37 were further away from the MIC, indicating the importance of confirming reported antimicrobial findings.

The introduction of the plasmid variant *S. mutans* pVA8912 enabled the continuation of experiments without contamination. The variant was affected by cinnamaldehyde and chlorhexidine in a similar manner; however, propolis was indicated to be less effective. By characterising the growth rate as similar and this difference from the wild type with propolis, the continued use of the variant was possible.

A range of chewing gums were prepared containing different antimicrobial compounds at a range of concentrations. These antimicrobial chewing gums were analysed to characterise the different release rates of the actives from the gums when mechanically chewed for three different lengths of time. The efficacy of this diffusate, including the released actives on planktonic and biofilm state *S. mutans,* was then investigated.

Cinnamaldehyde had the largest percentage release from the chewing gum of all the agents tested, with 19% of the total added released. This was a fifth of the published MIC value; however, in the experiments conducted within this chapter, a complete bactericidal effect was observed at the fifteen-minute chew time. This indicates that the efficacy of cinnamaldehyde against planktonic *S. mutans* was significantly stronger than previously published. However, when tested against biofilm state *S. mutans,* 4X the MIC (4000 ug/ml) was required to see a reduction in the culturable count.

In the spectrometry data, propolis was shown to release poorly from the gum, with a maximum of 1% released during 15-minute chewing, equating to 0.0007 µg/ml propolis in the diffusate. Surprisingly, despite this minimal release, the diffusate achieved a complete bactericidal effect against planktonic *S. mutans*. This unexpected effect indicated a higher release of propolis than 1%; the fractions of propolis being released may have had a spectral peak different to the 326 nm peak which was investigated. A full spectral scan should be done on the diffusates to see if there

is a larger peak elsewhere; this would provide a more realistic release concentration. This may show the concentration of the bactericidal compounds. HPLC could be used in conjunction with this to find out which fractions of propolis are being released; these fractions could then be synthesized and used directly.

Most of the chlorhexidine chewing gums showed no release; this should be investigated further. Firstly, its incorporation into the chewing gum could be determined; by dissolving the chewing gum in dichloromethane; and then calculating the chlorhexidine concentration present using the spectrophotometer. The chewing experiments could then be completed again using water instead of PBS as this would remove any potential interaction between the chlorhexidine and the NaCl to understand whether the chloride level is hindering the release of the chlorhexidine from the gum. Secondly, the incorporation of chlorhexidine acetate, instead of the gluconate, into the chewing gum would allow the investigation to see if this salt form of chlorhexidine has improved release concentrations, as well as investigating if it is able to kill bacteria from contact without being release from the chewing gum.

The results in this chapter have shown that selection of antimicrobials for use in chewing gums needs to take into consideration a number of different factors. The actives solubility and MIC are two important factors that impact the release of these actives and the feasibility of their use in chewing gums, as shown with chlorhexidine and methyl salicylate. Another important factor should be flavour; cinnamaldehyde has a strong flavour, which, when incorporated at levels required to cause a bactericidal effect, may become unpalatable for the consumer. However, the quantity of cinnamaldehyde added to the 1X MIC equates to 1% of the chewing gum; there was an expired patent which uses 0.5% to 3% cinnamaldehyde in chewing gum (Cherukuri, 1987). This provides an insight into the acceptable quantity of cinnamaldehyde to add. These factors should be used to guide chewing gum antimicrobial choices in the future.

The effect of antimicrobial-incorporated chewing gums was determined to be an additive effect rather than the hypothesized synergistic effect. The antimicrobials were likely affecting the deeper layer of the biofilm; however, this did not increase the bactericidal effect beyond that of the treatments separately. The presence of these antimicrobials will be able to create a bactericidal effect on the biofilms out of reach of the chewing gums, such as in the gingival sulcus. This makes the use of antimicrobial chewing gums a benefit to oral hygiene routines and should help reduce oral biofilms.

Improving the release profile of active agents is the most important next step for the development of antimicrobial chewing gums. It would be valuable to explore different ways of

incorporating the actives, such as encapsulation and liquid centre-filled gums, to investigate if a higher release can be achieved using these approaches. One potential issue with using a faster release of the antimicrobial is with an increased salivary rate during chewing; the antimicrobial may be cleared from the oral cavity too quickly to have an effect. A workaround would be to have a fast-release portion of antimicrobial paired with a slower-release mechanism, such as a liquid-filled centre and gum base with added antimicrobials; this would only be possible if the antimicrobials have a mild flavour.

The experiments conducted in this chapter characterising the use of cinnamaldehyde and propolis as oral antimicrobials, were completed on *S. mutans.* Further experiments should be completed *in vivo* to investigate the community changes. Cinnamaldehyde has a strong flavour, so testing it *in vivo* would provide a mechanism to assess its antimicrobial effects as well as optimising formulations with a high level of consumer acceptability. The positive effects of cinnamaldehyde and propolis on *S. mutans* bacteria means these two compounds were taken forward into the next stages of experiments.

Chapter 4:

Biofilm analysis of the oral microbiome when exposed to antimicrobials and chewing and investigating DNA yield from oral biofilm extraction.

"I can never find the thing that does the job best until I find the ones that don't".

Thomas Edison

Chapter 4 Biofilm analysis of the oral microbiome when exposed to antimicrobials and chewing, and investigating DNA yield from oral biofilm extraction.

4.1 Introduction

Within this chapter, an *ex vivo* plaque and saliva microcosm will be developed. The number of possible bacterial species in the oral cavity exceeds 700 (Aas *et al.*, 2005). This means working with an *ex vivo* microcosm is the best way to gain insight into the oral cavity's biofilms and how it compares to the single species work done previously investigating, the single early coloniser, *S. mutans* in Chapter 2 and Chapter 3.

With the oral biofilm being comprised of such a wide range of species, the effects of antimicrobials on the biofilms will be varied. As observed in Chapter 3, the efficacy of antimicrobials on a specific species can also vary between laboratories. Therefore, between people, who have unique microbiomes, the antimicrobial efficacies may alter significantly. It is known that when colocalising with certain species, previously susceptible bacteria can become more resistant (Kara *et al.*, 2007; Tavernier *et al.*, 2017). The investigation of how this multi-species biofilm reacts to the previously tested antimicrobials is crucial, as the next step is to test their viability for inclusion in an oral antimicrobial product. Based on the data collected in Chapter 3, chlorhexidine, cinnamaldehyde, and propolis will be tested against the *ex vivo* microcosm.

The use of the previously developed model, as described in Chapter 2, will help to create a thick biofilm, with the use of an *ex vivo* microcosm as the inoculum, increasing the representivity of the system. The use of aerobic and aerotolerant culturable counts will help provide insight into where, if at all, the antimicrobials are having an effect. This is important as many of the disease-related species are present in the anaerobic pockets of the biofilms. This can be paired with CLSM to show the effects on the biofilm architecture.

ure analysis, sequencing could provide more insight into community changes. Previously the use of sequencing has been invaluable in discovering which species are present without having to rely on the culturing of species, due to the oral microbiome being the second largest human microbiome.

Whole genome sequencing and 16S sequencing are the most common techniques used to identify the species present. In general, for whole genome sequencing, between 100-1000 ng total DNA is

required (GenoHub, 2019). For Novogene 16S sequencing, the total DNA recommended is 200 ng; however, samples as low as 30 ng can be run with risk (Novogene, personal communication, 23rd November 2022). However, due to the huge number of species present in the oral microbiome, a higher quantity of DNA would provide a better representation of the community, improving the representativity of the species present in low numbers.

To extract the DNA from oral biofilms, common commercial DNA extraction kits, such as the PowerBiofilm kit from Qiagen, are used. These kits are heavily refined with chemicals and buffers specifically designed for DNA extraction and clarification with organic compound removal. Historically, a cruder method of phenol/chloroform DNA extraction was used; this relied on simpler chemical reactions with fewer steps.

When studying the oral biofilm using sequencing, PCR methods are often used (Edlund *et al.*, 2013; Li *et al.*, 2021; Sousa *et al.*, 2022). PCR is used to increase the quantity of DNA before sequencing by amplifying the DNA present, allowing for easier sequencing runs. PCR works by splitting the strands of DNA and synthesising two new complementary strands using Taq polymerase, thus doubling the quantity of the DNA. This can introduce bias as it is run for many cycles, usually between 25 and 35 cycles, and if there is more of one species' DNA than another's, this difference may be amplified with each round. This may lead to the more prevalent species being sequenced many times and some species being lost, but this change is hard to quantify as the presence and amplitude of the bias varies depending on which species are present (Brooks *et al.*, 2015). PCR also introduces bias based on the guanine-cytosine base content of the DNA; during PCR, a high GC content reduces the replication rate (Laursen, Dalgaard and Bahl, 2017). Based on the high potential for bias when using PCR, maximising the initial DNA quantity during the extraction process is an essential step to improve oral microbiome community profiling.

4.2 Chapter aims

The aim of this chapter was to create an *ex vivo* microcosm for the oral microbiome by collecting saliva and plaque samples for use as the inoculum.

The second aim was to use the *ex vivo* microcosm in the developed model to investigate the changes to the biofilms after antimicrobial exposure and exposure to mechanical chewing forces.

This next aim was to provide insight into how changing from a single-species biofilm to a multispecies community biofilm affects the response to antimicrobial and mechanical force exposures.

The fourth aim of this chapter was to characterise the changes in DNA concentration yielded from different extraction protocols.

The final aim of this chapter was to optimise the extraction of *ex vivo,* lab-grown oral biofilms for 16S sequencing without the use of PCR.

4.3 Methods

4.3.1 Saliva and plaque microbiome collection and stock creation

Fourteen volunteers provided saliva and plaque samples, following ethical approval (ERGO: 51936.A1) using informed consent. The inclusion and exclusion criteria are presented in Appendix E pg. 138. Volunteers did not brush their teeth for 24 hours before the sample collection and abstained from eating and drinking anything other than water for 2 hours prior to collection. They brushed their teeth with a sterile toothbrush, without toothpaste, for five minutes, spitting out regularly into a sterile Falcon tube; they then spat into the same tube until 5-10 ml of saliva was collected.

The head of the toothbrush was cut off and vortexed in 5 ml of PBS for 20 seconds. The PBS and saliva, and plaque samples were centrifuged separately at 14000 rpm for 10 minutes. The saliva was transferred to a Duran bottle, and the PBS was discarded, leaving behind the bacterial pellet. The collected bacteria from the toothbrush and saliva and plaque samples were then recombined and resuspended in 1 ml of PBS per volunteer; all the samples were then pooled and vortexed. The samples were pooled to get a greater cross-section of the bacteria present in the oral cavity. Glycerol stocks were created by the addition of 750 µl of the resuspended bacteria to 750 µl of 50% glycerol in a cryovial aerobically, and were stored at -80°C.

4.3.1.1 Culturable counts and confocal imaging for *ex vivo* biofilms exposed to chewing forces and antimicrobials

Supplemented BHI (sBHI) was made and sterilised; this contained BHI medium with haemin, vitamin K and hog gastric mucin (Sigma Aldrich). The BHI media with hog gastric mucin was sterilised using heat, whilst the vitamin K and haemin were sterilised using filtration. A CDC bioreactor containing hydroxyapatite discs and 350 ml of sBHI was sterilised and set on a stirring hot plate at 37°C, stirring at 60 rpm with a 0.3 ml/min flow rate. 800 µl of the *ex vivo* microcosm stock was added to the CDC bioreactor. The CDC bioreactor and media tank were covered with foil to prevent light from affecting the media or bacterial growth. The reactor was run for 120 hours before the discs were removed and processed.

One disc from each reactor was used as a control and exposed only to PBS, one from each reactor was exposed to cinnamaldehyde (1 mg/ml, 1X MIC), propolis (0.035 µg/ml, 1X MIC) or chlorhexidine (0.24%, 1X MIC). The next set of four discs per reactor were chewed using the manual chewing method (as described in 2.3.3.1), one chewing in the presence of PBS; three were

chewed in the presence of antimicrobials; cinnamaldehyde (1 mg/ml, 1X MIC), propolis (0.035 µg/ml, 1X MIC) or chlorhexidine (0.24%, 1X MIC). Each of these treatments lasted 15 minutes; after this, the discs were washed in PBS using a rocking motion and then transferred to a new sterile 12-well plate for scraping or staining. The discs were then scraped, and spot plated as previously described (2.3.2.1). The plates were then put in both aerobic and anaerobic incubators overnight. This provides aerobic and aerotolerant culturable counts.

The discs being used in confocal imaging were exposed to propidium iodide and SYTO9 stains before setting them in Mowiol, as previously described (2.3.2.1). Samples were imaged using the inverted Leica SP8 confocal laser scanning microscope. A 63X oil immersion lens was used with Argon and DPSS lasers, scanning sequentially at 1 µm intervals. These lasers are set at 488 nm and 561 nm, respectively, to excite the dyes. Five images were taken on each disc, in identical places on each sample, to avoid bias. Initially, to get to the correct focus, a FITC filter was used on the first sample site. Images were obtained using the LAS AF software.

4.3.1.2 Culturable counts for *ex vivo* biofilms exposed to chewing forces and antimicrobials after growth in a Zurich-based system

A sterile 12-well plate containing hydroxyapatite discs and 2 ml of 1/5th strength sBHI, and 100 µl of the *ex vivo* microcosm stock was added to each well. This was placed in the 37°C CO_2 incubator for 120 hours. Every 24 hours, 1 ml of broth was removed from the well, and 1 ml of fresh 1/5th BHI broth was gently added down the side of the well. After 120 hours, all the broth was removed from each well. This was replaced with 2 ml of either cinnamaldehyde (4000 µg/ml, 4X MIC) or propolis (0.14 µg/ml, 4X MIC) and the discs were exposed for 15 minutes to the antimicrobial with and without chewing. After this time, the biofilms were washed using 2 ml of PBS and a rocking motion.

A cell scraper was then scraped over the disc for 30 seconds, and 1 ml of PBS was then pipetted up and down onto the disc three times before 200 µl was removed and added to a 96-well plate. This was then serially diluted, and spot plated as previously described, before being incubated in both an aerobic incubator and an anaerobic incubator.

4.3.2 Growing *ex vivo* biofilms for DNA extraction

A 12-well plate containing hydroxyapatite discs and 2 ml of 1/5th strength sBHI, and 100 µl of the *ex vivo* microcosm stock was added to each well. This was placed in the 37°C CO_2 incubator for 120 hours. Every 24 hours, 1 ml of broth was removed from the well, and 1 ml of fresh 1/5th sBHI

broth was added gently down the side of the well. After 120 hours, all the broth was removed from each well, and the discs were washed in 2 ml of PBS.

4.3.3 Removal of biofilms from coupons and additional lysis steps

The first biofilm removal method tested was cell scraping, the wash was removed, and 1 ml of fresh PBS was added directly onto the biofilm. A cell scraper was then scraped over the disc for 30 seconds. The PBS was then pipetted over the disc three times before being added to a 1.5 ml Eppendorf tube.
The second biofilm removal method was using a bath sonicator (UltraWave). The biofilm discs were placed in 1 ml of PBS, then placed into the bath sonicator and sonicated for 15 minutes. The PBS was then pipetted over the disc three times before being added to a 1.5 ml Eppendorf tube. Then the samples were sonicated on ice for 120 seconds at 12% amplitude with 10 second pulses using the Fisherbrand™ 505 Sonicator with Fisherbrand™ FB4418 3.1 mm Microtip Probe.

The third biofilm removal method was cell scraping with an additional cell lysis step, the wash was removed, and 1ml of fresh cell lysis buffer (ethylenediaminetetraacetic acid (EDTA) (FisherScientific), Tris (FisherScientific) and sodium dodecyl sulphate (Merck) were added directly onto the biofilm. A cell scraper was then scraped over the disc for 30 seconds. The cell lysis buffer was then pipetted over the disc three times and left at room temperature for 5 minutes before being added to a 1.5 ml Eppendorf tube.

The fourth biofilm removal method was cell scraping with a cell lysis step and a freeze-thaw step. The cell scraping in PBS was repeated as before; after adding to the 1.5ml Eppendorf tube, it was put in the -20°C freezer until frozen solid before thawing back to room temperature.

The fifth biofilm removal method used was cell scraping in PBS with an additional mechanical lysis step using fine needle aspiration. The cell scraping in PBS was repeated as before; once scraped, the PBS was pulled into a syringe through a 27-gauge needle three times.

4.3.4 Power biofilm extraction method

The DNA was extracted from the *ex vivo* biofilms using a PowerBiofilm kit (Qiagen) according to the manufacturer's protocol. This DNA was then quantified using the Qubit 2.0 (ThermoFisher) and the Invitrogen dsDNA High-Sensitivity/Broad-Sensitivity working solutions and standards. The Qubit is a fluorometer, which measures the absorbance of a sample; the added dyes fluoresce brightly once bound to DNA.

4.3.5 Different extraction methods

A phenol/chloroform (Sigma-Aldrich) extraction was completed following the protocol from (Wright, Adelskov and Greene, 2017).

4.3.6 Effect of different coupon material

Ex vivo biofilms were grown on three different coupon types, a hydroxyapatite coupon (Biosurfaces Technology), a plastic coupon (Biosurfaces Technology), and a glass coupon (Biosurfaces Technology).

4.3.7 Recovery of DNA from hydroxyapatite coupons

The cell scraping in PBS was repeated as before and once scraped, the PBS was transferred into a 1.5 ml Eppendorf and centrifuged (VWR Microstar12) at 13000 rpm for 10 minutes. The PBS was then removed, and the cells were resuspended in 1 ml of dH_2O. After two minutes, the tube was recentrifuged, the water removed, and 1 ml of fresh dH_2O added, and this was repeated a further two times.

Potassium phosphate buffer was made up of K_2HPO_4 (Sigma-Aldrich) and KH_2PO_4 (Sigma-Aldrich) at six concentrations doubling from 0.01 M to 0.32 M.

As a proof of concept experiment for the potassium phosphate buffer theory, planktonic *Listeria monocytogenes* Scott A (CECT) was obtained from a student within the laboratory as it was readily available immediately whilst the oral biofilms grow. *Listeria monocytogenes* Scott A (CECT) was collected from a BHI plate using a sterile loop. A hydroxyapatite disc was scraped using a cell scraper in 1 ml of PBS to release hydroxyapatite into the PBS. The *L. monocytogenes* Scott A was then added to this PBS containing hydroxyapatite, vortexed for 10 seconds and left for 5 minutes. The PBS was transferred into a 1.5 ml Eppendorf and centrifuged at 13000 rpm for 10 minutes. The PBS was then removed, and the cells were resuspended in 1ml of the lowest potassium phosphate buffer concentration for 5 minutes, then spun at 13000 rpm for 5 minutes and then exposed to increasing concentrations of potassium phosphate buffer consecutively for five minutes each, repeating the centrifuge step in between buffer exposures. This process was repeated using 5-day oral biofilms, the cell scraping in PBS was repeated as before.

Alongside the DNA quantification from the Qubit, these samples also had their purity assessed using the Nanodrop 2000 (ThermoScientific). The Nanodrop 2000 works using absorption, DNA and RNA (260 nm), proteins and contaminants (230 nm) absorb ultraviolet light at different peaks

allowing for the determination of the ratios and, from that the purity. A clean result would have a 260/280 ratio of 2 and a 260/230 of 1.8.

4.3.7.1 Statistical analysis

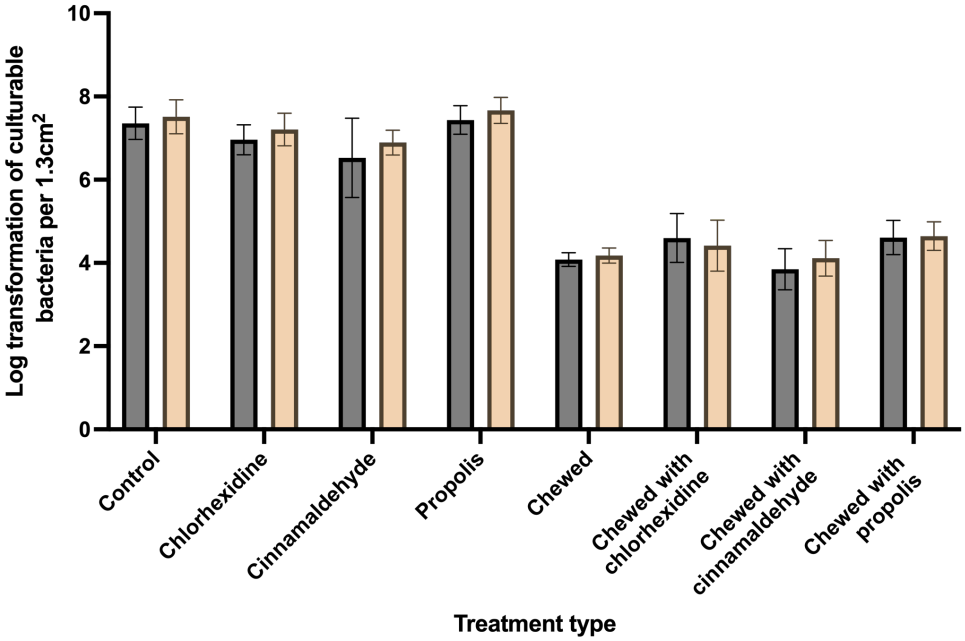
The statistical tests used in this chapter are stated in the figure legends of each figure. The main statistical test used was ANOVA. GraphPad Prism 9 was used to run the statistical analyses. In all statistics, a significance level of $P<0.05$ was used, with all values above this being classified as non-significant. The culturable counts were log-transformed before the mean was calculated, with a SD.

4.4 Results

4.4.1 Investigation into the changes to the biofilm culturability, viability and architecture

4.4.1.1 Culturable counts for *ex vivo* biofilms exposed to chewing forces and antimicrobials

The next stage in increasing the representivity of this model was to use an *ex vivo* oral saliva and plaque microcosm as the inoculum. These *ex vivo* biofilms were then exposed to antimicrobials and chewing forces, and chewing in the presence of antimicrobials. Figure 29Figure 30 shows there were no differences in the log transformation of the number of culturable bacteria from the control when the biofilms were exposed to 1X MIC antimicrobials for 15 minutes after 5-day growth in a CDC reactor. This was true for both the aerobic bacterial counts and anaerobic counts, with ANOVA P values shown in Table 4. The variability between biological repeats was minimal for the control, chlorhexidine and propolis; however, cinnamaldehyde has a larger variability increasing the standard deviation of the treatment. When the *ex vivo* biofilms were exposed to chewing, there was a 3.5-log decrease in the culturable bacterial count for the aerobic and anaerobic bacteria; this difference was significant. When the oral biofilms were exposed to antimicrobials whilst being chewed, there was no additional reduction in culturable bacteria



observed.

Figure 29: The log transformation of the number of culturable *ex vivo* bacteria after growth for 5-days in a Centre for Disease Control bioreactor, in brain heart infusion broth supplemented with haemin, vitamin K and hog gastric mucin. These biofilms were then exposed to treatments for 15-minutes. These were chlorhexidine (0.2%), cinnamaldehyde (1000 μg/ml), or propolis (0.035 μg/ml), or exposed to manual chewing using the modified Wessel method, with and without chlorhexidine (0.2%), cinnamaldehyde (1000 μg/ml), or propolis (0.035 μg/ml). Mean with 1+/- standard deviation plotted.

Table 4 Analysis of variance results for the significance difference of the treated biofilms to the control and analysis of variance results for the significance of difference of the chewed in the presence of antimicrobials to the chewed control. *Ex vivo* oral biofilms grown in supplemented brain heart infusion in a centre for disease control bioreactor for 5 days. P values based on the data presented in Figure 28.

Treatment	Aerobic	Anaerobic
Control vs Chlorhexidine	P= 0.8798	P= 0.8716
Control vs Cinnamaldehyde	P= 0.2671	P= 0.2828
Control vs Propolis	P= 0.9997	P= 0.9951
Control vs Chewed	P= 0.0001	P= 0.0001
Control vs Chewed with chlorhexidine	P= 0.0001	P= 0.0001
Control vs Chewed with cinnamaldehyde	P= 0.0001	P= 0.0001
Control vs Chewed with propolis	P= 0.0001	P= 0.0001
Chewed vs Chewed with chlorhexidine	P= 0.3985	P= 0.8313
Chewed vs Chewed with cinnamaldehyde	P= 0.8547	P= 0.953
Chewed vs Chewed with propolis	P= 0.3827	P= 0.4348

As no difference was observed when exposed to 1X MIC, a higher concentration was tested. In Figure 30Figure 33, statically grown *ex vivo* biofilms were exposed to a 4X higher concentration of antimicrobials either when chewed or not. A significant decrease was observed, with a 3-3.5-log decrease in culturable bacteria observed in the chewed samples; regardless of the antimicrobial presence, all three had a significance of P=0.0001 compared to the control, no chewed sample. When exposed to the antimicrobials alone, there was a small decrease for cinnamaldehyde, but a small increase for propolis, neither of which were significant (P=0.16 and P=0.20, respectively).

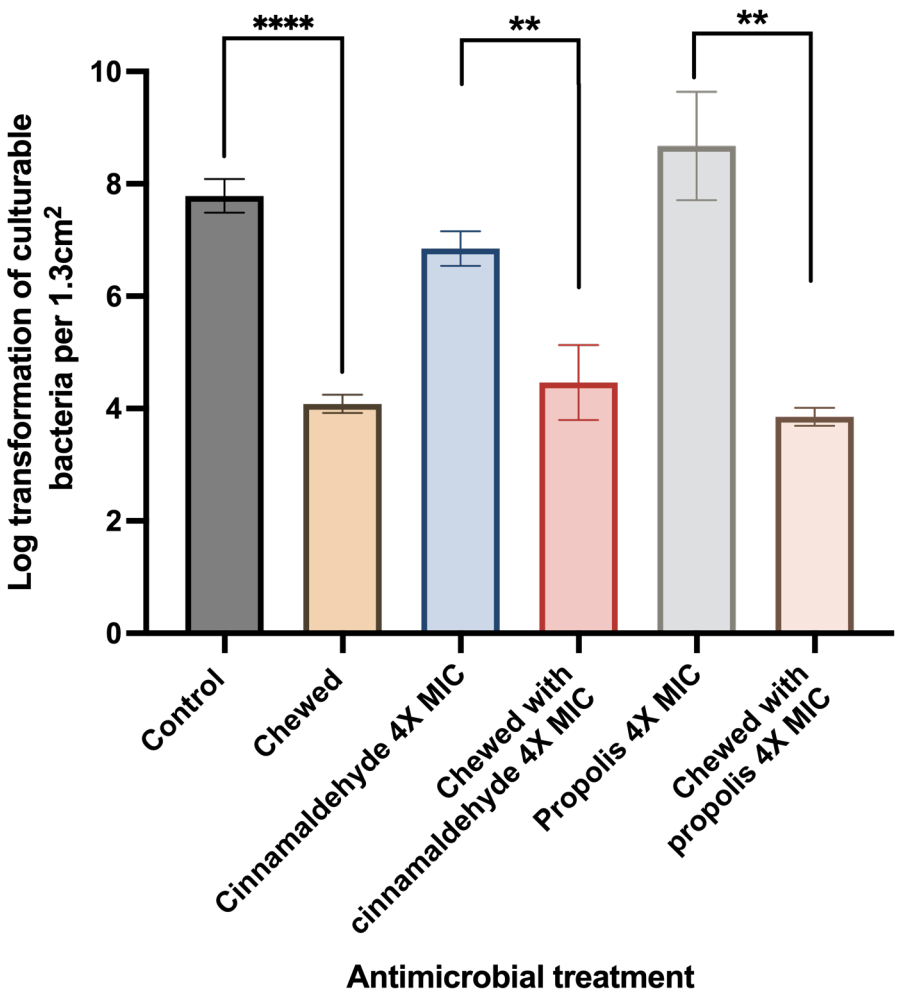


Figure 30: The log transformation of the number of culturable *ex vivo* bacteria after growth for 5 days in a static Zurich-based model in brain heart infusion supplemented with hemin, vitamin K and hog gastric mucin. These biofilms were then exposed to treatments for 15-minutes; these were cinnamaldehyde (4000 μg /ml), or propolis (0.14 μg /ml), or exposed to manual chewing using the modified Wessel method, with cinnamaldehyde (4000 μg /ml), or propolis (0.14 μg /ml). Mean ± 1 standard deviation, n=3. *= p=0.05, **= p=0.01, ***= p=0.001, ****= p=0.0001.

4.4.1.2 The investigation of *ex vivo* biofilm viability and structural changes

4.4.1.3 Confocal images

In addition to the culturability counts, confocal images were taken for each treatment type for the *ex vivo* biofilms. The confocal images shown in Figure 31 comprise of a representative image of the control disc (A), a biofilm exposed propolis 0.035 μg/ml for fifteen minutes (B), and a biofilm chewed in the presence of cinnamaldehyde 1000 μg/ml (C). This selection of treatments should show a range of effects.

Figure 31A shows a thick, healthy biofilm with an undulating architecture, with a mix of live and dead bacteria was present. Figure 31B show thick healthy biofilms after exposure to propolis at 0.035 μg/ml, 1X MIC. There was a low percentage of dead cells with little variation in biofilm thickness. Figure 31C showed a confocal image of the *ex vivo* biofilm after being exposed to chewing forces in the presence of cinnamaldehyde. There was a large section of the biofilm removed, with much of the remaining biofilm stained dead, with a few pockets of live cells. In this image, the hydroxyapatite disc was visible as it auto-fluoresces green. Due to the staining and autofluorescence, the top layer of the biofilm is well stained; then, there is a gap of unstained biofilm before the disc. This is most easily observed in Figure 31C.

In addition to the representative images in Figure 32 and Figure 32 shows a representative image from reactor 2 biofilms after exposure to propolis at 0.035 μg/ml. This was highlighted as it shows a significantly different biofilm architecture. There were hyphae structures forming a mycelium, indicating a high presence of fungi, with the remaining biofilm having a smooth appearance.

4.4.1.3.1 Representative images of 5-day biofilms

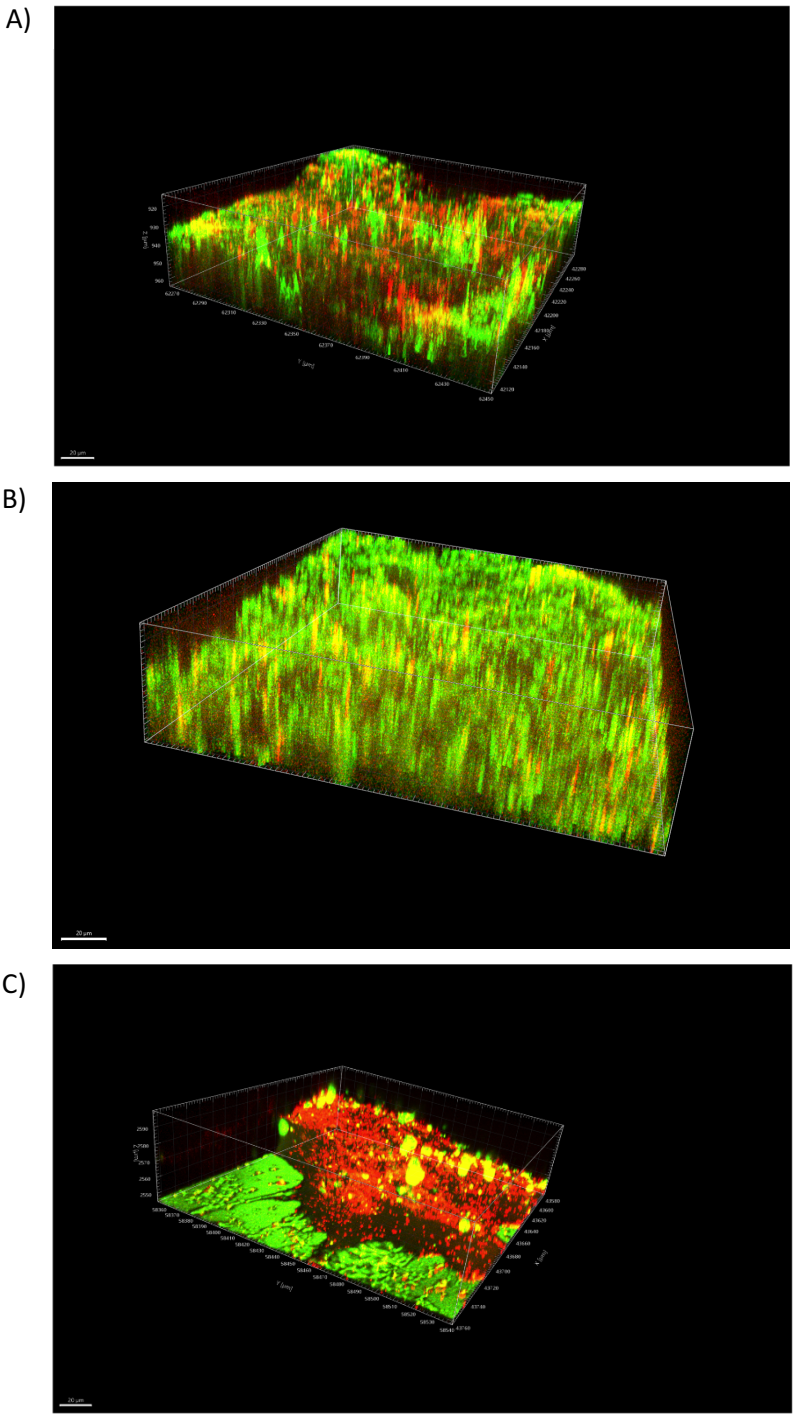


Figure 31: Shows three images of *ex vivo* biofilms, A is a representative image of the control disc, B is a representative image of a biofilm exposed propolis 0.035 μg/ml for fifteen minutes, C shows a representative image of a biofilm chewed in the presence of cinnamaldehyde 1000 μg/ml. *Ex vivo* biofilms were grown in a Centre for Disease Control reactor in brain heart infusion broth supplemented with haemin, vitamin K and hog gastric mucin. After growing for 5 days they were treated, then stained with LIVE/DEAD stain and imaged on a confocal laser scanning microscope, 63X oil immersion lens. The scale bar shown is 20 μm, the green base is the hydroxyapatite disc.

4.4.1.4 Image showing potential fungal presence

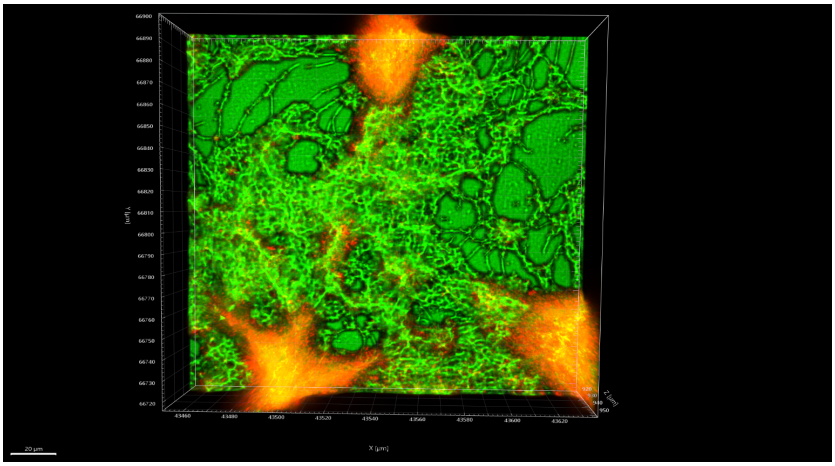


Figure 32: Shows a top-down image of *ex vivo* biofilm, an image of a biofilm exposed propolis 0.035 μg/ml for fifteen minutes. *Ex vivo* biofilms were grown in a Centre for Disease Control reactor in brain heart infusion broth supplemented with haemin, vitamin K and hog gastric mucin. After growing for 5 days they were treated, then stained with LIVE/DEAD stain and imaged on a confocal laser scanning microscope, 63X oil immersion lens. The scale bar shown is 20 μm, the green base is the hydroxyapatite disc.

4.4.1.4.1 Data analysis of confocal images

To interrogate the confocal images, image analysis was used, it was possible to characterise the percentage of cells which stained green, indicating live cells, the maximum thickness of the biofilm and the percentage of the disc covered. Three reactors were run, with five images per treatment type, the means of this data is plotted in Figure 33. This provided insight into if the biofilm was healthy and if the overall architecture of the biofilm had been affected by the treatment.

When the three biological repeats of the CDC bioreactors were combined, there was a high level of variation. This means that over the three characterized outputs and eight treatment types, no treatments had a statistically meaningful effect, as seen in Table 5. This large variation observed between biological repeats, indicates optimisation may be required to improve repeatability. This may be in the form of model optimisation or improving the microcosm collection and cell cluster disruption. Whilst the reactors could be investigated separately and differences could be found (see appendix F pg. **Error! Bookmark not defined.**), this would not be statistically significant as they would only be N=1.

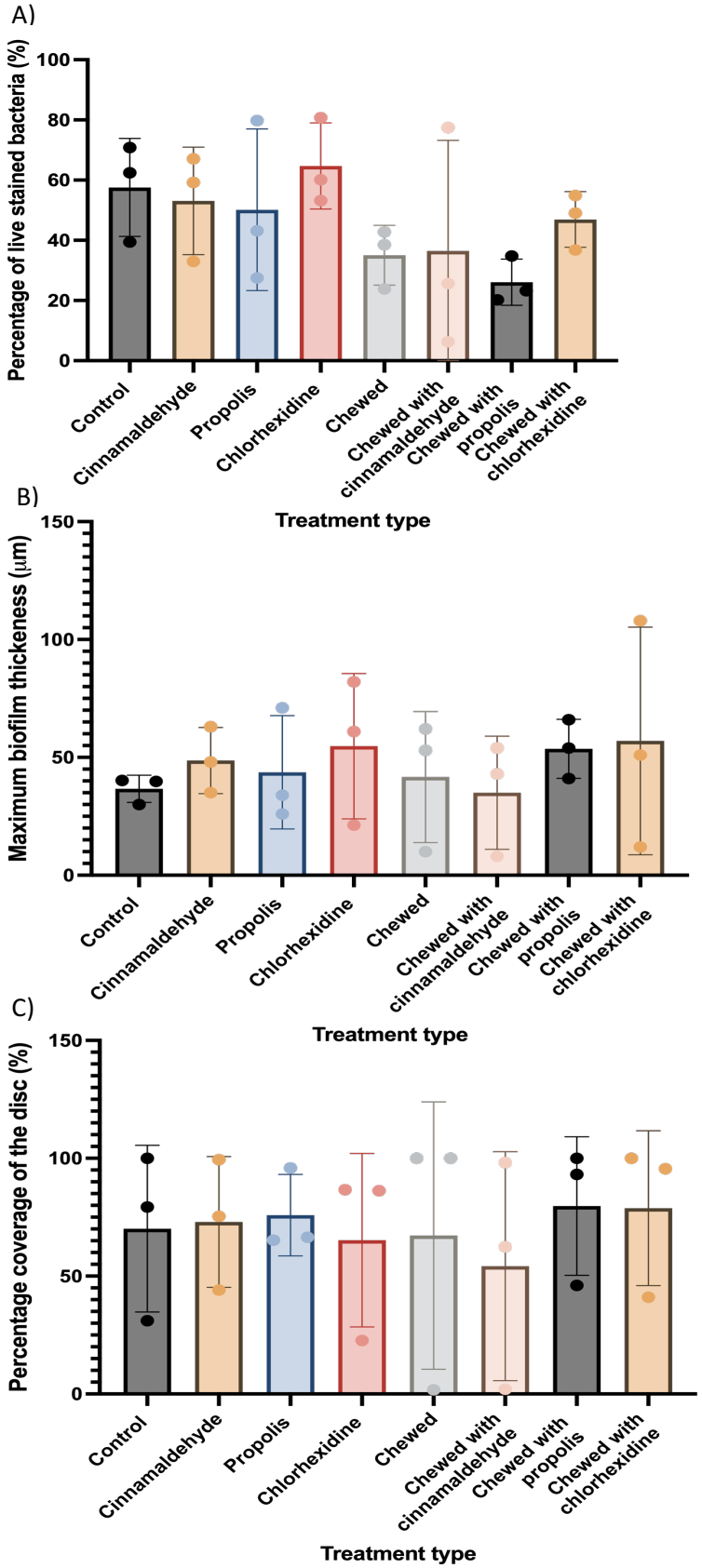


Figure 33 A, B and C: showing the percentage of live stained bacteria (A) maximum biofilm thickness (B), and percentage coverage (C) The IMARIS LIVE/DEAD image analysis of confocal laser scanning microscopy images is plotted. *Ex vivo* biofilms were grown for 5-days in a centre for disease control bioreactor in brain heart infusion supplemented with hemin, vitamin K and hog gastric mucin. These biofilms were then exposed to treatments for 15-minutes, these were chlorhexidine (0.2%), cinnamaldehyde (1000 g/ml), or propolis (0.035 g/ml), or exposed to manual chewing using the modified Wessel method, with and without chlorhexidine (0.2%), cinnamaldehyde (1000 g/ml), or propolis (0.035 g/ml). These biofilms were then stained with LIVE/DEAD stain and imaged on a confocal laser scanning microscopy. The biofilm z-stacks were then analysed in IMARIS to produce percentages of green-stained bacteria compared to red-stained bacteria. Mean ± 1 standard deviation, n=5. * = significance of difference from the control. *= p=0.05, **= p=0.01, ***= p=0.001, ****= p=0.0001.

Table 5: Analysis of variance of treated samples compared to the control sample. The significantly different results from the control are highlighted in red for the significant decrease. Analysis of variance was calculated using GraphPad prism 9.

Treatment	Percentage of live stained bacteria	Maximum biofilm thickness	Percentage disc coverage
Cinnamaldehyde	0.9996	0.9907	0.9999
Propolis	0.9957	0.9995	0.9997
Chlorhexidine	0.9973	0.9263	0.9998
Chewed	0.5959	0.9997	0.9999
Chewed with cinnamaldehyde	0.6540	0.9999	0.9932
Chewed with propolis	0.2789	0.9438	0.9996
Chewed with chlorhexidine	0.9755	0.8813	0.9996

4.4.2 Characterising different biofilm removal techniques

The next step into determining the changes to the microbiome was to use sequencing for each reactor individually. To investigate the changes in the species present DNA extraction and sequencing was attempted. A disc from a CDC bioreactor, which had run for 5 days with an *ex vivo* microcosm inoculum, had the DNA extracted from it using the PowerBiofilm kit and manufacturers protocol. This created a DNA concentration of 0.90 ng/µl. This concentration of DNA was too low for sequencing.

To ensure the biofilm was being fully removed from the discs, other removal techniques were tested on statically grown 5-day *ex vivo* microcosm biofilms. The DNA concentrations from these five other methods are outlined in Table 6. The cell scraping from the statically grown biofilm produced a higher DNA concentration of 1.51 ng/µl compared to a decrease to 0.86 ng/µl when using sonication. Further steps were added to the cell scraping to improve the collection of the biofilm and adding a cell lysis buffer and freeze-thaw step created the largest increase to 3.01 ng/µl.

Table 6: Comparison of different mechanical removal methods for *ex vivo* oral biofilms on hydroxyapatite discs. The deoxyribonucleic acid concentration (ng/⌈l) is shown from these 5-day *ex vivo* biofilms after six types of mechanical removal and Qiagen PowerBiofilm extraction kit protocol. N=1.

Samples	DNA concentration ng/µl (Qubit)
From a CDC bioreactor– cell scraped	0.90
Static – cell scraped	1.51
Static – sonicated	0.86
Static – cell scraped with lysis buffer	1.40
Static – cell scraped with lysis buffer and freeze-thaw	3.01
Static – cell scraped with lysis buffer and fine needle aspiration	2.01

4.4.3 Alternative extraction protocols

To see if the concentration of DNA could be increased using a different extraction protocol a phenol/chloroform extraction protocol was tested. The extraction of a 5-day *ex vivo* oral biofilm created an increased DNA extraction of 2.56 ng/µl. A bead beater step was added to the phenol/chloroform extraction, which improved the DNA concentration to 5.12 ng/µl, as shown in Table 7. The addition of the PowerBiofilm DNA clean-up steps reduced the DNA concentration to 0.24 ng/µl and 0.71 ng/µl with the addition of the PowerBiofilm DNA clean-up steps and bead beating.

Table 7: The deoxyribonucleic acid (DNA) concentration (ng/ μ l) when a phenol/chloroform extraction protocol is used. Three additional optimisation steps were tried, using a bead beater mechanical lysis for 1 minute, the addition of PowerBiofilm DNA clean-up steps and both the mechanical lysis and clean-up steps.

Samples	DNA quantity ng/µl (Qubit)	Standard deviation
Phenol/chloroform	2.56	0.11
Phenol/chloroform with a mechanical lysis step	5.12	3.88
Phenol/chloroform with the PowerBiofilm clean steps	0.24	0.12
Phenol/chloroform with the PowerBiofilm clean steps and a mechanical lysis step	0.71	0.58

4.4.4 Investigating the impact of altering the growth surface

The next step to investigating the low DNA concentration was to see how different substrates for the biofilm, changes the DNA concentration collected. *Ex vivo* biofilms were statically grown for 5 days on different coupon materials; hydroxyapatite and glass discs had similar DNA concentrations of 1.51 ng/µl and 1.54 ng/µl, respectively, as shown in Table 8. The plastic coupon had a much higher DNA concentration of 14.04 ng/µl. The plastic coupon DNA extraction had a large variation between coupons ranging from 8.00 ng/µl to 26.80 ng/µl.

Table 8: Comparison of different coupon materials for static growth of 5-day *ex vivo* oral biofilms. The deoxyribonucleic acid concentration (ng/ μ l) is shown from these biofilms after cell scraping and PowerBiofilm extraction kit protocol. Glass and plastic N=3, hydroxyapatite N=1.

Samples	DNA quantity ng/µl (Qubit)	Standard deviation
Hydroxyapatite	1.51	N/A
Glass	1.54	0.29
Plastic	14.04	10.33

4.4.5 Recovering DNA from hydroxyapatite binding

4.4.5.1 Use of Listeria monocytogenes Scott A

The discovery that hydroxyapatite binds DNA and can be released using increasing concentrations of potassium phosphate buffers, led to the proof of concept experiment using for *L. monocytogenes* Scott A. Figure 34 shows the DNA recovery for *L. monocytogenes* Scott A from hydroxyapatite. There is an upward trend for DNA concentration as the concentration of potassium phosphate buffer increases, with 0.16 M potassium phosphate buffer creating the highest DNA concentration. With this higher starting bacterial cell count, there is a 93% recovery of DNA concentration from the hydroxyapatite. The purity achieved with the *L. monocytogenes* is also much higher, with averages of 1.9 for the 260:280 ratio and 2.2 for the DNA: organic material ratio.

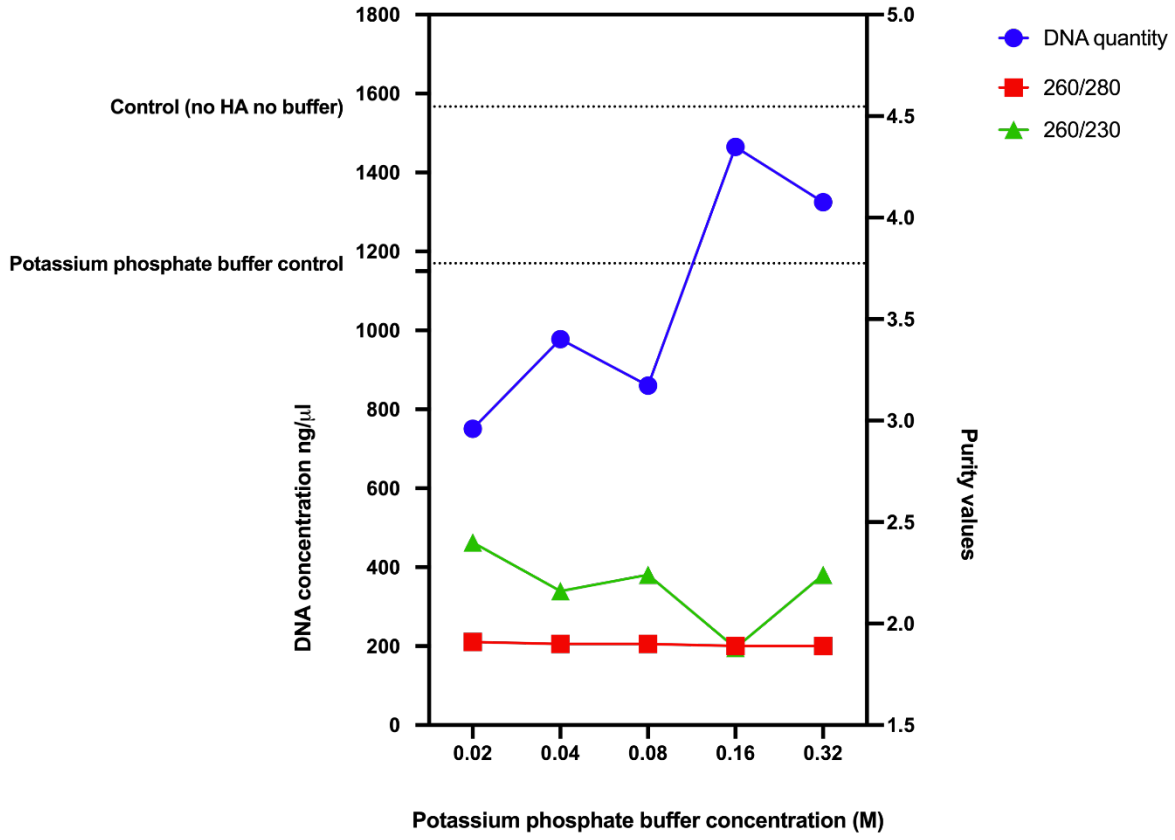


Figure 34: Deoxyribonucleic acid (DNA) concentration (blue) (ng/ μ l) from *Listeria monocytogenes* Scott A taken of a brain heart infusion agar plate and resuspended in five ml of phosphate buffered saline and hydroxyapatite. These bacteria were then exposed to potassium phosphate buffer and had the DNA extracted using the PowerBiofilm protocol. The purity of the DNA is plotted on the right y-axis, 260/280 shown in red is the ratio of DNA to RNA, and the 260/230 shown in green is the ratio of DNA to organic compounds. The dotted line at 1567 ng/ μ l shows the extracted DNA without exposure to hydroxyapatite or potassium phosphate buffer. The dotted line at 1170 ng/ μ l shows the extracted DNA after exposure to potassium phosphate buffer but no hydroxyapatite. N=1.

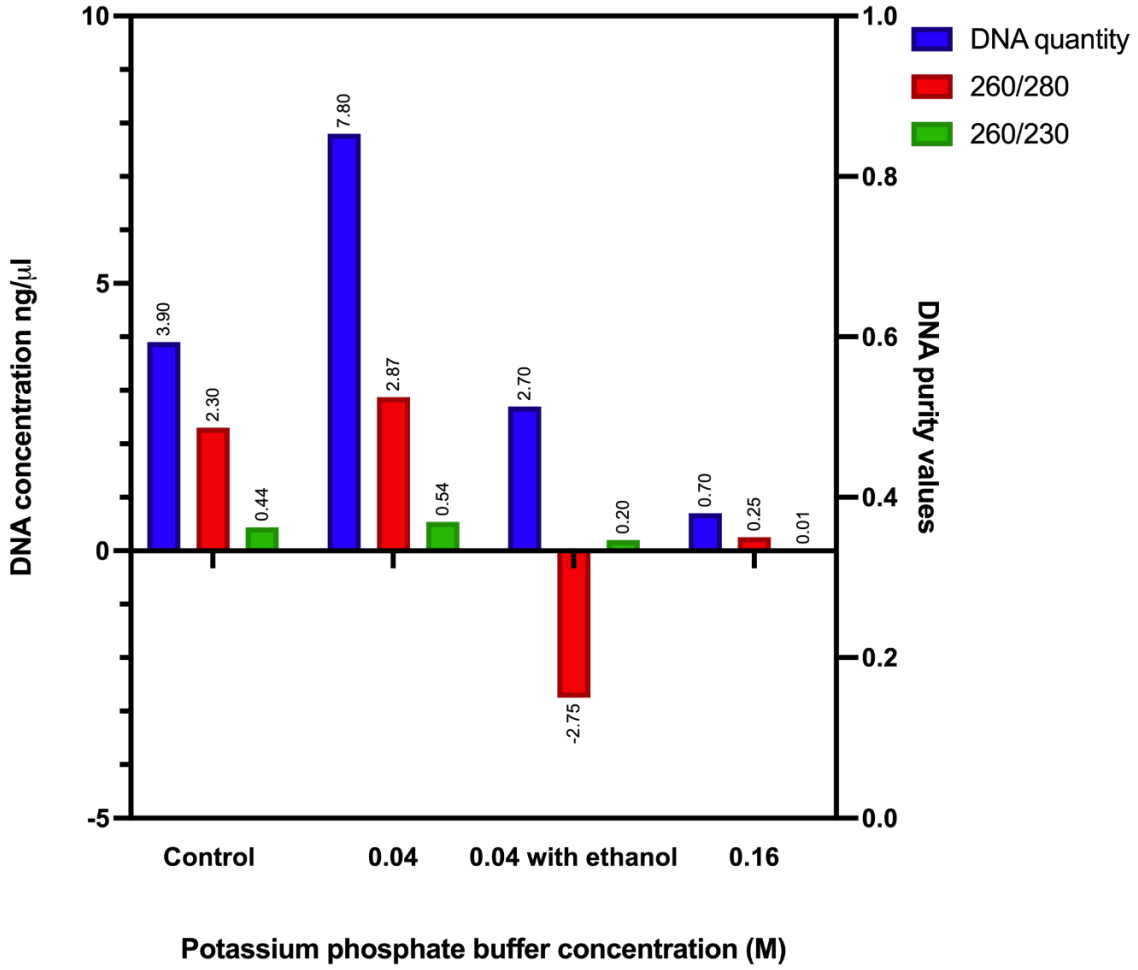


Figure 35: Deoxyribonucleic acid (DNA) concentration (blue) (ng/ μ l) from 5-day, statically grown oral biofilms after exposure to increasing concentrations of potassium phosphate buffers after being cell scraped off a hydroxyapatite disc. These bacteria then had the DNA extracted using the PowerBiofilm protocol. The purity of the DNA is plotted on the right y-axis, 260/280 shown in red is the ratio of DNA to RNA, and the 260/230 shown in green is the ratio of DNA to organic compounds. N=1.

Based on the promising results from the *L. monocytogenes* Scott A experiment, this was recreated using a 5-day *ex vivo* oral biofilm. Using a potassium phosphate buffer, the DNA recovery was mixed; at 0.04 M a high recovery of 7.80 ng/µl was achieved; however, the added ethanol cleaning step reduced the DNA recovery to 2.72 ng/µl. When the bacteria were exposed to further higher concentrations of potassium phosphate buffers, the amount of DNA extracted was reduced to 0.70 ng/µl as seen in Table 9. Figure 35 shows the purity of the DNA was low, with the ratio of DNA to organics compounds maintaining around 0 and the DNA to RNA ratio varying from -2 to 2, indicating low purity. The use of a water washing step increased the oral DNA concentration to 6.65 ng/µl as shown in Table 9.

Table 9: Deoxyribonucleic acid (DNA) concentration (ng/ μ l) of two different recovery methods for DNA from hydroxyapatite. A repeated wash step using H_2O after cell scraping, and the use of a series of increasing concentration potassium phosphate buffers, once completed with an additional ethanol clean step. N=1.

Samples	DNA quantity ng/µl (Qubit)
Washing in H_2O	6.65
Potassium phosphate buffer 0.04 M	7.80
Potassium phosphate buffer 0.04 M with an ethanol clean	2.72
Potassium phosphate buffer 0.16 M	0.70

4.5 Discussion

4.5.1 Investigation into the changes to the biofilm culturability, viability and architecture

The data presented in this chapter aimed to elucidate any effects that may have occurred when the *ex vivo* biofilms were exposed to chewing forces and antimicrobials. Previous chapters investigated the effects of antimicrobials and chewing forces on a single species, *S. mutans* biofilm (Chapter 3). This will provide insight into how the presence of multiple species affects the efficacy of antimicrobials and chewing forces.

4.5.2 Culturable counts for *ex vivo* biofilms exposed to chewing forces and antimicrobials

Based on the data presented in Figure 29, the addition of chewing influenced the culturable counts. In each of the chewed samples, regardless of the antimicrobial presence, a similar significant decrease was observed. This 3.5-log decrease in culturable cells was observed in both the aerobic and aerotolerant counts, potentially due to the aerobic processing of the samples. The presence of antimicrobials caused no further decrease in the culturable count of the bacteria. A similar effect was observed when the biofilms were exposed to a higher concentration of antimicrobials; the chewing caused a reduction in the culturable count, whilst the addition of antimicrobials did not (Figure 30). It was hypothesized that this was due to the presence of many different species; antimicrobials are known to work on certain species of bacteria but do not commonly kill all species or at the same concentration. For example, cinnamaldehyde does have antifungal effects against *Candida albicans*, a fungus commonly found in the oral cavity; however, this effect was only present at significantly higher concentrations, 62.5 µg/ml (Pootong, Norrapong and Cowawintaweewat, 2017). This was also true for propolis which was found to have a MIC of 1-12 µg/ml for oral microbes (Gebara, Lima and Mayer, 2002).

4.5.3 The investigation of *ex vivo* biofilm viability and structural changes

When investigating the effects of the three biological repeats of the 5-day, *ex vivo,* CDC bioreactor-grown biofilms, there were only two significant changes across the three investigated image analyses. This was due to the high level of variation between each reactor, this was apparent when investigating every image from each reactor.

The samples collected from participants were pooled and vortexed, it was hypothesized that when aliquoted into separate freezer stocks, there was a small level of difference, and as each reactor used 800 µl of inoculum, a different aliquot was used each time. It was hypothesized that

within the reactors, different species became prevalent over the time course of the experiment and that this would have the capability to alter biofilm structure and response to antimicrobials and shear stresses.

Figure 31 A shows a representative control disc biofilm, the biofilm appears to be healthy with an undulating structure, and a similar structure was seen after propolis exposure in Figure 31B. Then comparing it to Figure 31C, the chewed with cinnamaldehyde disc, this treated disc visually had a much higher percentage of dead cells with a large patch of biofilm removed. In this image, the chewed with cinnamaldehyde has had a large effect; however, among the other technical repeats, more variation was observed.

Within the images taken from reactor 2, in the two with the lowest average maximum thickness, propolis and chewed with cinnamaldehyde, a mycelium-like structure was observed, this is shown in Figure 32. Hyphae structures were visible across the hydroxyapatite discs, with the biofilm taking on a smoother appearance. Based on this smoothness, it was hypothesized the fungus present was *Aspergillus*, which produces a smooth covering EPS (Fanning and Mitchell, 2012). A Schiff's stain could be used to confirm the presence of fungi.

No fungi were observed in the images from the other reactors, however it cannot be ruled out from being present without further more in-depth tests. If there was a higher level of fungi present, it would affect antimicrobial efficacy and which species could join the biofilm as it appears to be one of the early colonisers. Cinnamaldehyde and propolis are known antifungal agents (Wang *et al.*, 2019; Sokolonski *et al.*, 2021); therefore, they were likely to have a higher effect on this biofilm compared to the other reactors.

The high level of variation occurring between the reactors, from the biofilm structure and the response to treatments, highlights the variation which can occur between different people and within the reactors. Even though the samples were pooled, differences were apparent between reactors, potentially based on which bacteria were able to colonise first. This helps to provide insight into how antimicrobial chewing gums would work in real-world situations. However, this does cause an issue with finding which antimicrobial chewing gums would be effective. One way to improve the reliability of the results would be to complete more biological repeats; a higher number of repeats would provide clarity on which are most effective, even with the high variability.

The confocal imaging shows a high level of variability, which has, up till this point, been attributed to the multi-species nature of oral biofilms. However, this variability was not seen with the CFU counts in aerobic or anaerobic conditions. A secondary hypothesis to this variability could be the

random sampling of the confocal images. Taking the images in the same places on each disc ensures there was no sampling bias occurring. However, this does not mean that the images were representative of the whole biofilm or disc. By taking five images per disc, each with an area of 40000 µm, the total percentage of the disc imaged was only 0.16%. With this level of the disc being imaged, it is hard to ensure that a representative area was imaged. One way to improve the imaging would be to look over the whole disc visually and take five images that were deemed representative. Whilst this increases the risk of bias, it could increase the representivity of the disc and potentially reduce variation.

This section would be incomplete without the mention of potential VBNC cells. The CFU counts show a decrease in culturability; however, the confocal images do not reflect that change; by definition, this could be due to the presence of VBNC cells. Whilst this is possible, due to the short amount of time between exposure and imaging, this altered state is unlikely. With no standardised method currently to distinguish VBNC cells from culturable cells, this hypothesis is difficult to prove either way. In addition to VBNC cells, persister cells are known to also form in biofilms, these cells are dormant and form spontaneously or in response to antimicrobials. These cells are able to withstand antimicrobials to a higher level than regular cells in the biofilm (Suppiger *et al.*, 2020).

There are only a few papers currently available for VBNC present in the oral cavity. *Staphylococcus aureus* is found in the oral cavity as well as within food processing lines (McCormack *et al.*, 2015). It has been characterised as being able to enter a VBNC state (Cheng *et al.*, 2023). Another paper hypothesizes *P. gingivalis,* a leading oral pathogen, with the potential to enter the VBNC state (Progulske-Fox *et al.*, 2022). Also, other *Streptococcal spp.* have been observed to have phenotypes and genes similar to those in and needed for the VBNC state, including their resuscitation from the VBNC state using resuscitation-promoting factors (Ramamurthy *et al.*, 2014). Biofilms have been documented as having subpopulations entering a VBNC state (Pasquaroli *et al.*, 2013).

It has previously been observed that VBNC cells have a higher tolerance to mechanical stresses, such as sonication (Weichart and Kjelleberg, 1996). Whilst gaining tolerance from, and being induced by, are not the same thing, it could be hypothesized that the cells may be entering the VBNC state to gain protection from the chewing gum. In addition to this, cinnamaldehyde has been observed as inducing the VBNC state in *S. aureus* when exposed to 0.5-1 mg/ml cinnamaldehyde for 1-3 hours (Cheng *et al.*, 2023). The experiments conducted within this chapter had a shorter exposure time of 15 minutes. There was also no significant difference

between the chewed and chewed in the presence of cinnamaldehyde; these factors do not indicate that the presence of cinnamaldehyde had an effect on the state of the cells.

4.5.4 Characterising different biofilm removal techniques

This chapter elucidates how different mechanical and extraction methods alter DNA yield for sequencing. Table 6 shows that a higher DNA concentration is observed from the static biofilm. This may be due to two reasons; the first is that the CDC bioreactor sample was stored in DNA protect (Cambridge Technologies) for two weeks before the DNA was extracted, and whilst this should not have decreased the DNA concentration, it should be considered. The second is that static biofilms were seen to have a higher CFU count compared to CDC-grown biofilms (2.4.1.1); in 3-day CDC bioreactor biofilms, 2.5 log fewer culturable bacteria were observed. Having fewer bacterial cells present will reduce the DNA concentration from the *ex vivo* oral biofilms.

Cell scraping was shown to be more effective than sonication at removing the biofilm with nearly double the DNA concentration, this may be due to using a suboptimal protocol. The addition of further lysis steps improved DNA concentration. This is likely due to these steps weakening the bacterial cells and making the breaking open of the bacterial cells in the PowerBiofilm extraction protocol more effective. The addition of the freeze-thaw steps was the most effective; this increase in DNA concentration is likely due to the formation of ice crystals in the bacterial cells; these crystals damage the bacterial cell walls weakening them (Huebinger, 2018).

4.5.5 Characterising different biofilm extraction techniques

A phenol/chloroform extraction protocol was used because it contained more intensive lysis steps. Using the phenol/chloroform extraction alone increased DNA extraction to 2.56 ng/µl, as seen in Table 7. To increase the cell lysis further, a mechanical bead beater lysis step was added, which doubled the DNA extracted. The final step to increase concentration was to pair the phenol/chloroform extraction with PowerBiofilm DNA clean-up steps; however, this decreased the concentration significantly, regardless of the additional mechanical lysis steps. This reduction may be due to the addition of unnecessary filtering steps causing DNA loss at the filters.

4.5.6 DNA recovery from hydroxyapatite

Hydroxyapatite discs are often used in oral biofilm growth as hydroxyapatite is found within teeth and therefore is the most representative surface for the bacteria to bind to. Table 8 shows the hydroxyapatite and glass discs had a similar DNA concentration; however, the plastic disc caused

an increase in DNA recovery to 14.04 ng/µl. Hydroxyapatite was found to bind DNA; as the cells are being scraped off the hydroxyapatite disc, the top layer of hydroxyapatite is also being removed. Then, as the cell walls are disrupted, the loose hydroxyapatite can bind to the free DNA and is removed during the cleaning stages of the extraction. Glass coupons also had a very low DNA concentration; it is hypothesized that this is due to reduced biofilm formation, glass is a harder surface for bacteria to colonise, as observed in (Haney *et al.*, 2018). This is due to bacteria having an increased binding affinity to rougher surfaces (Sherry Zheng *et al.*, 2021).

Literature states that hydroxyapatite binds DNA and proteins strongly (Kemp and Smith, 2005). This strong bond was used in HPLC to pull DNA or proteins out of solutions by attaching hydroxyapatite to a column. Potassium phosphate buffers were then poured through these columns at increasing concentrations to release the DNA and proteins from the hydroxyapatite and back into solution (Kawasaki, Takahashi and Ideda, 1985). Based on this, DNA recovery from the hydroxyapatite using a series of potassium phosphate buffers was investigated.

A proof-of-concept experiment was run using *Listeria monocytogenes* Scott A bacteria from a streak plate. A large loopful of bacteria was resuspended in PBS and aliquoted into seven Eppendorf's, five of which had hydroxyapatite added to them. The DNA concentration from the control extraction with no buffers or hydroxyapatite was 1567 ng/µl when exposed to the buffers without hydroxyapatite, a DNA concentration of 1170 ng/µl was achieved, this indicated the presence of the buffers do reduce DNA concentration. However, when hydroxyapatite is added, and the sample is only exposed to 0.02 M potassium phosphate buffer, the concentration diminishes to 751 ng/µl. A positive trend is then seen as potassium phosphate buffer concentrations increase, with the highest concentration achieved at 0.16 M recovering the DNA to 1465 ng/µl. This is higher than the potassium phosphate buffer control and a recovery of 93.5% of the total DNA. Throughout these concentrations, the purity of the DNA remained high in relation to both the ratio of 160:280 and 260/230; this indicated that the extraction of DNA using this protocol works well and can produce concentrations with very pure DNA.

Based on the previous positive result, this was repeated using oral biofilms grown statically for five days before being cell scraped into PBS. The control sample had a DNA concentration of 3.9 ng/µl, then when exposed to the first two concentrations up to 0.04 M potassium phosphate buffers, a DNA concentration of 7.8 ng/µl was achieved. However, when an ethanol clean was added to help purify the DNA before the PowerBiofilm extraction, the DNA concentration lowered to 2.7 ng/µl. Indicating the DNA may not have precipitated out fully, or the ethanol was not fully removed during the DNA extraction. Then when exposed to concentrations up to 0.16 M potassium phosphate buffers, a DNA concentration of 0.7 ng/µl was achieved. Across the tested

concentrations, the purity of the DNA for DNA: RNA is very mixed with a very low purity at 0.16 M. The DNA: organic compound ratio, however, was poor throughout, maintaining a value around 0. The low DNA concentration may be due to the starting quantity of DNA being very low. If the potassium phosphate buffers work by outcompeting the DNA to bind to the hydroxyapatite, the low initial quantities of DNA may reduce the potassium phosphate buffers' efficacy due to the high quantity of available hydroxyapatite.

In Table 9, the cell-scaped biofilms were washed with dH_2O, which increased the DNA concentration to 6.65 ng/µl; this method was simpler than the potassium phosphate buffers; however, it yielded a slightly lower concentration of DNA.

The two most effective changes observed were growth on a plastic coupon and exposure to phosphate buffer at 0.04 M; however, both are still too low for 16S sequencing. Combining two or more of these methods may be beneficial in achieving a high enough DNA quantity for sequencing without combining reactors or using PCR.

4.6 Conclusions and Further work

The investigation into antimicrobials and chewing forces on *ex vivo* biofilms provided information on bacterial removal by chewing forces. When investigating the CFU counts of the *ex vivo* bacteria when exposed to chewing, a clear and significant decrease was observed; this was observed in both the static and continuous culture methods, as well as for both aerobic and aerotolerant species. This decrease indicates a strong removal effect had occurred due to the mechanical forces of chewing, reflecting what was observed with the single species experiments. However, when investigating this significant difference using confocal imaging, this change is not seen.

A person's oral microbiome is as unique as their fingerprint (Edlund *et al.*, 2017); and can continuously change, this has been reflected in the data presented in this chapter. When the three CDC bioreactor runs were combined as biological repeats, the variation was so high no significant conclusions could be drawn from it. However, if separated out, conclusions could be drawn, but with an N number of one, no significant conclusions could be stated.

Three hypotheses were discussed as to why there was such a high level of variation across the reactors. The strongest hypothesis was that the differences observed between reactors regarding variation within each disc, antimicrobial response, and resilience to the shear forces associated with chewing was based on microbial community changes between reactors. It is known that different species form different types of biofilms, with the colocalization of species also altering biofilm properties. From that, it could be inferred that there are significant differences in the microbial community. This could be investigated using 16S sequencing. Using 16S sequencing on the initial inoculum, then on the control, and each of the treatment types could provide valuable insight into the efficacy of the model for maintaining species diversity as well as the community effects of each of the treatments. The addition of ITS sequencing to 16S would also help to identify the fungal species which are predicted to be present in reactor 2. Combining both sequencing types; 16S and ITS would provide a well-defined oral microbiome. By incorporating a propidium monoazide step, dead cells could be removed before PCR allowing for sequencing only living cells in the biofilm.

Alongside the 16S sequencing, further investigation into how the age of the biofilm affects how it responds to antimicrobials and chewing forces would be beneficial. As parts of the biofilms in the oral cavity are removed by brushing and other hygiene steps, there will be many different stages of biofilms present in the oral cavity. This could be investigated by testing on biofilms from 1 day to 7 days old. Seeing how each treatment affects the different stages of the biofilm could provide valuable insight into when the treatment is most beneficial.

The static growth of a 5-day oral biofilm produced a higher DNA concentration than a 5-day oral biofilm grown in a CDC bioreactor. This is comparable with the data shown in 2.4.1.2, where a higher CFU count was observed in the statically grown biofilms. Sonication of the biofilms did not improve DNA concentration, indicating that the physical removal of the biofilm from the disc is not causing the low DNA concentration, or that the sonication may breakdown the DNA.

The change to the phenol/chloroform extraction rather than the PowerBiofilm was tested as the phenol/chloroform extraction uses a simplified list of reagents which limits the potential for unintentional reactions whilst being known for its intensive cell wall disruption. The highest DNA concentration from this extraction technique was 5.2 ng/µl, which was achieved with an additional mechanical lysis step. Whilst this is a large improvement in concentration, it was still too low to be used in sequencing.

The surface the biofilms were growing on was then tested, and it was found that plastic coupons provide a significantly higher DNA concentration than hydroxyapatite or glass. Plastic provides the highest DNA concentration during extraction; however, it is not the most representative surface for the biofilms to grow on. The plastic coupon created such a drastic improvement compared to the hydroxyapatite; it was indicated that something was interfering with the DNA release from the hydroxyapatite. This led to the discovery that hydroxyapatite binds strongly to DNA and proteins.

Washing the scraped cells with dH_2O increased the DNA concentration to 6.65 ng/µl; this exposure to water and centrifuging created a significant increase with minimal changes to the extraction protocol. Potassium phosphate buffers were used to recover the DNA from hydroxyapatite, and this allowed for the second-highest DNA concentration, after plastic coupon use, with a concentration of 7.8 ng/µl. This indicates that the DNA is present in the solution, but further processing is needed to release it to a state where it could be sequenced.

This chapter aimed to investigate methods to improve DNA concentration from *ex vivo* oral biofilms, specifically those grown on hydroxyapatite discs. Several potential improvements to DNA concentration have been detailed through the phenol/chloroform extraction with a mechanical step and the exposure to potassium phosphate buffers showing significant improvements. The combination of some of the steps discussed should be further characterised to see if their improvements are synergistic and create a concentration high enough for sequencing without PCR.

The use of a plastic coupon rather than hydroxyapatite created the largest increase in DNA concentration. The bacterial attachment to plastic may be different from the bacterial attachment

to hydroxyapatite. This could be studied further by carrying out sequencing on three samples; multiple hydroxyapatite discs combined, one hydroxyapatite disc with PCR, and a plastic disc. Each of these methods has its drawbacks; combining multiple reactors may be problematic due to the variation seen across reactors in 4.4.1.2.1. PCR may cause bias in which species are amplified and lead to the loss of diversity in the microbiome. Finally, the use of plastic may alter which species attach and which are capable of co-localization. By sequencing all three and comparing them, a true characterisation of these effects could be mapped.

The final section of further work that could be carried out is to repeat the potassium phosphate buffer exposure, using 0.16 M potassium phosphate buffer in place of the 1 ml of PBS during the cell scraping. It is hypothesized this may bind the hydroxyapatite as it is released from the disc preventing any DNA from binding. The later exposure to increasing concentrations could be used as necessary.

Chapter 5:

Conclusions and further work.

Chapter 5 Conclusions and further work.

The aim of this thesis work was to provide a greater understanding of the interaction between mechanical forces occurring within the oral cavity and the oral microbiome, as well as to improve the understanding of the impact of combining the mechanical disruption of chewing with a simultaneous release of antimicrobial agents on simulated biofilm plaque, with an aim to use it to improve oral health.

Over half the global population (56%) experience oral health diseases during their lifetime and the follow-on life-changing, systemic diseases which can occur after. Finding novel removal mechanisms and combining previously used methods together can be used to help reduce oral biofilms and improve oral disease.

With this in mind, three key steps were completed; the first was to develop an oral biofilm model incorporating a mechanical chewing action. The second was to use this novel model to test potential oral antimicrobials on single species planktonic bacteria and biofilms. This projects investigations were finalised using the novel model and most promising oral antimicrobials against a created *ex vivo* saliva and plaque microcosm.

5.1 Development of a novel oral biofilm model

The first step in this project, was the development of an oral biofilm model which incorporates the mechanical actions involved in chewing gums. Chewing gum experiments are often executed *in vivo* or using planktonic bacteria. This novel model allows biofilms to grow in the CDC bioreactor flow system before being exposed to the mechanical forces of chewing gums via the E1000 indenter or the modified Wessel method.

This model was validated and compared to the Zurich model for growth. It was observed that the CDC bioreactor has a lower number of culturable bacteria within the biofilms. However, the biofilm proved to be more resilient to mechanical shear forces when grown in the reactor. It was hypothesized that this was due to the flow of media the biofilms are exposed to in the CDC bioreactor. This shear flow will encourage a thicker EPS, creating a more robust biofilm.

This model modified the bacterial exposure to chewing gum from the Wessel method (Wessel *et al.*, 2015), and it characterised the bacterial removal from chewing gum when in a biofilm state. It was observed that when using the manual modified Wessel method, there was no significant difference from the E1000 indenter, mechanical method. This means that when testing small

quantities of biofilms, the modified Wessel method could be used, but when processing large quantities of biofilms, the E1000 indenter would be more appropriate. It was hypothesized that this would help maintain accuracy throughout the experiments as the prolonged use of the manual chewing method would lose accuracy through human error.

5.2 The identification of potential oral antimicrobials

Using the developed model to create an oral biofilms model chapter 3, investigated a pool of potential oral antimicrobials. Three of these key antimicrobials, cinnamaldehyde, methyl salicylate and propolis, were observed to have a strong potential as antimicrobial as gum additives. Chlorhexidine was used as a comparative control as it is an industry standard. Cinnamaldehyde was determined to have a bactericidal effect at 3750 ppm against *S. mutans,* whilst propolis and methyl salicylate had a bacteriostatic effect at 0.7 ppm and 160000 ppm, respectively. As *S. mutans* is an early coloniser of the oral cavity and has cariogenic properties, this effect shows they may be a helpful additive to oral hygiene regimes when incorporated into chewing gums.

5.3 The development and characterisation of antimicrobial chewing gums

Cinnamaldehyde, methyl salicylate and propolis were all highlighted as good potential oral antimicrobials for incorporation into chewing gums due to their bacteriostatic and bactericidal effects. However, due to the MIC value of methyl salicylate, it was not possible to incorporate it into chewing gum at an effective concentration. Cinnamaldehyde, propolis and chlorhexidine were all successfully incorporated into chewing gum formulations.

These antimicrobial chewing gums were then mechanically chewed, and their diffusates were analysed to determine the quantity of released compound. The observed release rates of all the antimicrobials were much lower than anticipated, with cinnamaldehyde having the largest release rate at only 19.37% of the total added antimicrobial. Propolis only has a maximum release rate of 0.95% of the total added antimicrobial. This small quantity of released propolis, however, did cause a complete bactericidal effect on planktonic *S. mutans.* This indicated either a higher concentration was released but showed a spectral peak at a different point or the 0.95% which was released contained highly antimicrobial properties.

Over the three time points, no chlorhexidine was detected. It was hypothesized that the lack of release of chlorhexidine was due to its molecular structure when in the gluconate salt form. This

large molecule will bind well to the long-chain polymers present in the chewing gum base. The highest release rates occurred after fifteen minutes of chewing, demonstrating that chew time had an impact on the amount of antimicrobial released. At these released concentrations, three of the chewing gums were able to inhibit planktonic *S. mutans'* growth.

The selection of which antimicrobials should be used in chewing gums is important. The molecular structure, weight and charge, and MIC are all important factors which determine release rates and feasibility of the chewing gums, as shown with chlorhexidine and methyl salicylate, respectively. This should be used to guide chewing gum antimicrobial choices in the future.

5.4 The investigation into the combination of antimicrobial presence during the mechanical forces of chewing

It was characterised in Chapter 3 that cinnamaldehyde was more effective at reducing the bacterial CFU count in an *S. mutans* biofilm when in the presence of chewing than when it was exposed to biofilms alone, and this effect was suggested to be additive rather than synergistic.

When *S. mutans* biofilms were exposed to 1000 µg/ml cinnamaldehyde, it had no significant effect ($P>0.05$). However, when cinnamaldehyde was exposed to the biofilm with the mechanical forces of chewing present, there was a significant reduction in the number of viable bacteria compared to when chewing alone ($P<0.05$). This indicates that cinnamaldehyde was having a significant effect. It was hypothesized this was due to the chewing mechanism lifting or breaking the biofilms and allowing the cinnamaldehyde to penetrate deeper. This suggestive additive effect is very promising for the development of antimicrobial chewing gums.

5.5 Using an *ex vivo* microcosm to provide a more representative response to the combined antimicrobial and mechanical effects

To improve the representivity of this model the data in Chapter 4 characterised the effects antimicrobials and chewing forces have on *ex vivo* oral biofilms. Culturable counts were performed on 5-day *ex vivo* biofilms from CDC bioreactor runs, using a range of treatments from antimicrobial exposure (with chlorhexidine, cinnamaldehyde and propolis) to the exposure of chewing forces and chewing in the presence of these antimicrobials. They indicated that at the tested concentrations, 0.24% (2.4 mg/ml), 1 mg/ml, and 0.035 µg/ml, respectively, the antimicrobials had no effect on the multispecies community in either aerobic or anaerobic species. However, the shear forces associated with chewing created a significant decrease in

bacteria, reducing the culturable counts by 3.5-log; this was true for both aerobic and aerotolerant counts. When antimicrobials were added to these chewing forces, no further reduction in culturability was observed. The seven treatments tested showed a relatively tight cluster for the biological repeats in the culturable counts, but this was not seen in the confocal images.

When investigating the confocal images for the 5-day *ex vivo* biofilms after the same treatments, a high level of variability was observed, both between biological repeats and sometimes within a disc. Whilst this variability should not have been seen due to the pooling of samples, it was highly representative of the oral cavity, as people have unique oral microbiomes, which continually change. Within the reactors, a clear trend was not discernible. With the CFU counts, a clear decrease in culturability was observed when exposed to chewing forces, but this was not supported by the image analysis. Over the three image analyses, percentage of live stained bacteria, maximum biofilm thickness and percentage coverage of the disc, there were no trends within the reactors, with each reactor responding differently to the other.

When viewing the images, a few interesting discs were found, the first being the exposure to propolis in reactor 2. This showed a fungi mycelium structure across the base of the disc; this was observed across a few of the treatments in reactor two, indicating this biological repeat had a strong fungal presence. For the chewed in the presence of cinnamaldehyde, reactor 1 showed a large area of biofilm removed whilst leaving behind other sections of untouched biofilm. This indicated that the chewing gum had pulled off a section of the biofilm.

There were three hypotheses as to why the image analysis did not agree with the culturable counts or show distinct trends. The first was the multispecies nature of the oral microbiome; it is possible that the inoculated reactors all deviated in the number and type of bacteria present after 5 days. The large number of species present then will all respond to the antimicrobials and chewing forces differently. In addition to this, certain species binding to each other can cause changes to the biofilm structure. This would explain why all three reactors had such varied responses. The second hypothesis was that the imaging was not representative of the biofilm. With five images taken randomly to avoid bias, only 0.16% of the disc was imaged. By chance, this could have caused the collected images not to be representative of the whole biofilm. One way to mitigate this would be to scan the whole disc to gain an understanding of the biofilm and then take representative images.

The final hypothesis was the presence of VBNC cells; the data presented in this chapter saw the culturable counts decrease, whilst the image analyses did not indicate a decrease in biofilm

viability or structure. The inducement of VBNC cells is thought to occur over a longer period of time; however, the VBNC state has been shown to increase tolerance to mechanical stress. Any of these hypotheses, individually or in combination, could have caused the effects observed.

5.6 Improving *ex vivo* oral biofilms DNA yield for use in 16S sequencing without using PCR amplification

The use of sequencing in the discovery of microbes in the oral cavity was essential for a full understanding since at least a third of the species present are non-culturable. 16S sequencing has been used frequently for this, using samples taken straight from the oral cavity. However, when growing the *ex vivo* microcosm inoculum into biofilms within the lab, PCR was often used to increase the DNA yield to a concentration acceptable for 16S sequencing, around a concentration of 30 ng/µl.

Chapter 4 also investigated different methods to improve DNA yield without using PCR. Hydroxyapatite discs are commonly used in oral biofilm experiments; this is because they have some of the binding motifs present on teeth, making them one of the most representative options for growing an oral biofilm. However, hydroxyapatite is known to bind strongly to DNA and proteins, reducing DNA yield from extractions, and in Chapter 4, a yielded concentration of only 1.51 ng/µl was observed. In general, phosphate buffers can be used to recover DNA from hydroxyapatite. However, due to the low initial yield of DNA, a high recovery was not observed in oral biofilm DNA extraction, an increase to 7.8 ng/µl was observed. It was hypothesised this was due to the potassium phosphate not being able to out-compete the DNA due to the high availability of hydroxyapatite in the system. This hypothesis was supported by the fact that there was a greater recovery of the DNA when the initial DNA concentrations were much higher.

Washing the hydroxyapatite and biofilm pellet in water increased the yield from 1.51 ng/µl to 6.7 ng/µl. Changing the extraction kit from the QIAGEN power biofilm kit to a phenol/chloroform method, which was hypothesised to have a stronger cell lysis step, only increased the DNA yield to 5.2 ng/µl.

The change that created the largest increase in DNA yield was using a plastic coupon rather than a hydroxyapatite coupon. This increased DNA yield to 14 ng/µl, and while this was a lot closer to an acceptable amount for 16S sequencing, a higher level would be beneficial for sequencing the bacteria that are only present in low levels due to the oral microbiome being a massive-multispecies microbiome. The use of hydroxyapatite was chosen to increase the representivity of the oral cavity, as the hydroxyapatite is the most similar surface to a tooth, so biofilm attachment

should be comparable in this model and *in vivo*. However, this representativity is at the expense of being able to sequence without using PCR.

5.7 Further work

The difference in biofilms when in the static and continuous model could be further characterised by investigating the matrix. Adding a matrix stain, such as SYPRO RUBY which stains proteins in the matrix, to the LIVE stain would allow for the visualisation of the cells inside the matrix and characterise how thick the EPS is and the cell density of each biofilm (Ravaioli *et al.*, 2020). Another step to further characterise the model would be to investigate how using a shaking incubator may improve the resistance of a statically grown biofilm to shear forces, such as chewing.

The low antimicrobial release concentrations from the chewing gum should be investigated in more depth; the first step for the chlorhexidine chewing gum would be to ensure it was incorporated properly in the first place. This could be investigated using dichloromethane to dissolve the chewing gum and then the spectrophotometer to determine the chlorhexidine levels. This will indicate if the chlorhexidine didn't incorporate into the mixture or if it is getting trapped due to its molecular structure. Further investigation into the release of propolis should also be completed by using a spectral scan on the chewing gum diffusate; it would show if other peaks were present, which may indicate a different release concentration for other propolis fractions.

Other release mechanisms could be used for the antimicrobials in the chewing gum, for example, using a liquid-filled centre or encapsulated granules of antimicrobials in the chewing gum. These types of release could be investigated to characterise if they improve the release rate of the chewing gums. If the release rates of the antimicrobials could be improved, they would be more efficient against oral bacteria in the planktonic and biofilm states.

A final consideration for antimicrobial chewing gums is their flavour. Cinnamaldehyde has a strong flavour, so testing it *in vivo* would show if it were palatable for the consumer as well as provide validation of its antimicrobial effects against the oral microbiome community. Previous cinnamon chewing gums had shown an inclusion of 0.5-3% of cinnamon to the total gum weight (Cherukuri, 1987). This is a positive indication as it is higher than our tested gum concentrations.

In Chapter 3, we observed a suggested additive effect between chewing and cinnamaldehyde exposure; further, more in-depth testing to prove whether the effect is additive or synergistic could be done to clarify the responses observed. Secondly, testing the mechanical chewing action in the presence of other antimicrobials, such as propolis and chlorhexidine, to investigate if the improved additive effect is seen with all antimicrobials or only antimicrobials with a specific mechanism of action could be carried out.

To add more depth to the results observed for antimicrobial and chewing on an *ex vivo* oral biofilm in Chapter 4, 16S sequencing should be completed. This would allow a greater knowledge of how the community profile changes over the course of the developed model and the treatments the biofilms are exposed to. This insight would help develop the understanding of how the community is changing with exposure to certain antimicrobials, providing insight into which areas of the biofilm are being removed, reduced or unaffected.

In Chapter 4, the difficulties of sequencing *ex vivo* oral biofilms grown on hydroxyapatite discs without using PCR are described in detail. Further investigations are needed to determine if a combination of the tested techniques could work to improve DNA yield, such as the use of the washing steps before the phenol/chloroform extraction. Or using the potassium phosphate buffer at the time of cell scraping to neutralise the hydroxyapatite as it is released and before the DNA is exposed. Finally, comparing *ex vivo* oral biofilms grown on plastic and *ex vivo* oral biofilms grown on hydroxyapatite discs with the use of PCR could provide valuable insights. Two key points are how the PCR could be introducing bias into the community and how the use of plastic as a substrate may affect which bacteria initially colonised the disc and how that affects the biofilm community.

5.8 Concluding statements

The work provided here is the basis for a new oral biofilm model which incorporates chewing. It is a well-defined model in which to investigate the effects of chewing gums on oral biofilms, and how the incorporation of antimicrobials may improve their efficacy.

These data show how *ex-vivo* biofilms are more resilient to antimicrobials than single species *S. mutans* biofilms. However, chewing gum is capable of a high removal rate in both single and multispecies oral biofilms. This project also optimised the DNA extraction from oral biofilms and a method has been outlined with three options to improve yield, with the potential combination of washing and phenol-chloroform extractions providing a higher yield whilst still using the hydroxyapatite disc without PCR.

Data shown here provides insights into promising natural antimicrobials for the use of reducing oral biofilms, and by extension, oral diseases. The further work proposed has the potential to develop the model into a very effective tool to study the effects of antimicrobial chewing gums on biofilms and guide future gum development.

Appendix A - Investigation of reoccurring contamination within laboratory experiments.

In the second year of my PhD, I began to experience issues with contamination. About half of my experiments were contaminated with visible, turbid growth or CFUs of a contaminating organism. This contamination continued for two years until I swapped to using *S. mutans* pVA8912, and the plates and broth containing erythromycin. This reduced my data output significantly and slowed down my progress.

There was no pattern as to when the experiment would get contaminated. The contamination could occur in the well plates, media or on the agar plates, and this would change with each repeat of the experiment. This contamination would outcompete *S. mutans*.

Initially, I created new broths, agars, and tips re-autoclaving everything; this did not reduce the contamination. I then autoclaved my pipettes and reduced my broth from a supplemented broth to plain BHI broth. There was still no improvement. I then investigated if it was the MSC hood that I was working in and tried different MSCs and working by a flame, as well as moving to a different bench. I swabbed the air vents above my bench and incubated them at 37°C overnight, but I did not find the source of the contamination. I tested the autoclave to ensure it was running at the correct temperature for the correct length of time.

My stocks were created from ATCC freeze-dried stocks and then made up of sterile BHI and 50% sterile glycerol. All experiments were started with a streak plate where one colony was isolated, therefore, the overnight should be pure. All media was streaked out at the time of use, and each well plate well was streaked out daily to check for contamination. The contamination appeared to form a pellicle on top of the BHI broth in the well. When plated out, it had large pale-yellow colony morphology. Using Gram-staining and light microscopy, I discovered it was a Gram-positive rod-shaped bacterium.

PCR tests were done using the primers in Table 10, using an *S. mutans*, gtfB, primer to check that it was contamination and not a novel colony morphology. A Quick-Load purple 1 kb plus DNA ladder was placed in the first well, then one blank well next to it. In the third well a water, negative control was used, then five different *S. mutans*/potential contaminations were checked. The fourth, fifth and sixth wells all show a band around 517 kb, which is a *S. mutans* band. All the wells show other bands, with common other bands at 1.5 kb and 2.5 kb, showing clear contamination.

Table 10: Primer sequences used in contamination polymerase chain reaction checks.

Primer	Primer sequence
S. mutans FWD	5’ ACTACACTTTCGGGTGGCTTGG
S. mutans RV	5’ CAGTATAAGCGCCAGTTTCATC

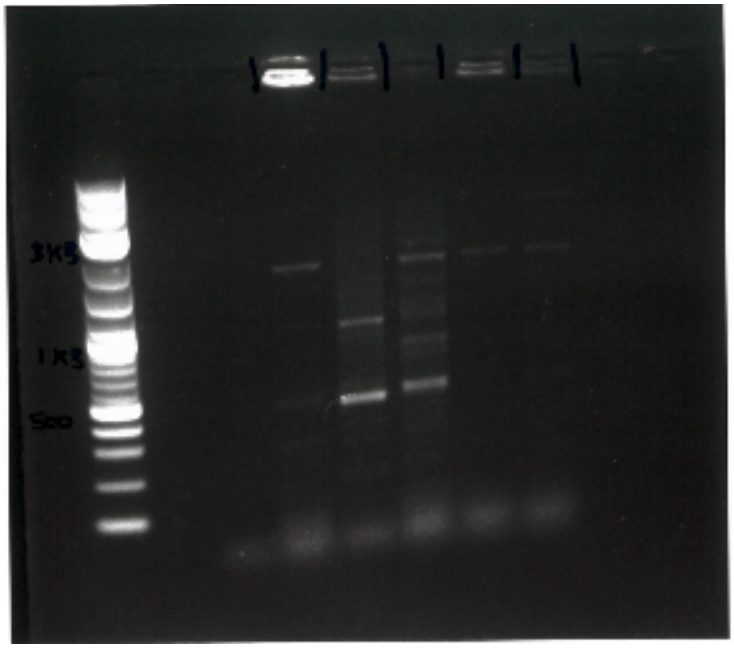


Figure 36: Image of a polymerase chain reaction gel, with a Quick-Load purple 1kb plus deoxyribonucleic acid ladder in the first well. The third well has a water control, fourth is believed to be *S. mutans*, fifth and sixth wells are contamination, and seven and eight are streak plates of *S. mutans*. *Streptococcus mutans* should have a band at 517b.

A contaminated overnight sample was sent to Youseq for sequencing. The contamination was identified as the class of the bacteria *Bacillales.*

The source of the contamination could not be found, and due to the reoccurring nature of the contamination, an antibiotic-resistant strain of *S. mutans* was gifted from Professor N Jakuboviks and Dr Ahmed at the University of Newcastle. The genetically modified *S. mutans* UA159 with plasmid pVA8912 was used. It contained a GFP tag and erythromycin resistance (Banasb, 2015). This GM strain was provided by Professor Nick Jakubovics from the University of Newcastle. This strain allowed for the presence of erythromycin in all future experiments; no contamination was seen after this change.

Appendix B - ANOVA tests for Figure 17 for each antimicrobial concentration against its control.

Table 14: Statistical tests for Figure 17, one-way analysis of variance was completed for each antimicrobial concentration against its relevant control. The limit for the test is <0.0001. A p value of p=0.05 makes the difference from the control significant.

Antimicrobial	PPM	P value (from one-way ANOVA)
Chlorhexidine	300	0.9999
	600	0.9812
	1200	0.4121
	2400	0.3811
	4800	0.0476
	9600	0.0150
	19200	0.0049
	38400	0.0049
LL37	0.004625	0.3228
	0.00925	0.0994
	0.0185	0.5050
	0.037	0.2967
	0.074	0.4458
	0.148	0.0811
	0.296	0.2072

Methyl salicylate	3750	0.9997
	7500	0.6689
	15000	0.0308
	30000	0.1057
	60000	0.0597
	120000	0.0770
	240000	0.1955
	480000	0.0181
Eucalyptol	31.25	>0.9999
	62.5	0.9998
	125	0.9997
	250	0.9997
	500	0.9997
	1000	0.9995
	2000	0.9997
	4000	>0.9999
Cinnamaldehyde	125	<0.0001
	250	<0.0001
	500	<0.0001
	1000	<0.0001
	2000	<0.0001
	4000	<0.0001
	8000	<0.0001

	16000	<0.0001
Menthol	12.5	0.9938
	25	0.9142
	50	0.8199
	100	0.8307
	200	0.9908
	400	0.9750
	800	0.9947
	1600	0.9997
Cranberry	3.125	0.9948
	6.25	0.8508
	12.5	0.9477
	25	0.8159
	50	0.6848
	100	0.8462
	200	0.8387
	400	0.9975
Propolis	0.035	0.0345
	0.07	0.0296

Appendix C - Chewing gum compound concentrations.

Table 15: Antimicrobial compound concentrations in the chewing gums created.

Antimicrobial type	Gum type	Compound quantity added for a 500g chewing gum batch
Control	Control	N/A
Cinnamaldehyde	1/8th X MIC (125µg/ml)	0.625g
	1/4th X MIC (250 µg/ml)	1.25g
	1/2 X MIC (500 µg/ml)	2.5g
	1 X MIC (1000 µg/ml)	5g
Methyl salicylate	0.13 X MIC (3.9mg/ml)	20g
Chlorhexidine	0.4 X MIC (0.096%)	4.8g
	0.8 X MIC (0.192%)	9.6g
Propolis	1 X MIC (0.035 µg/ml)	0.175g
	2 X MIC (0.07 µg/ml)	0.35g

Appendix D - Spectroscopy and Calibration curve data.

Initially, a spectral scan was completed for each compound at a range of concentrations to determine the absorbance peak; an example is shown in Figure 41. If more than one peak was discovered, the peak which didn't cross with the ethanol control or gum base control was chosen. The range of concentrations was then used to create linear regressions for each compound. The chewing gum diffusates then had their absorbance read at this peak and compared to the linear regressions to determine the quantity of compound released Figure 24.

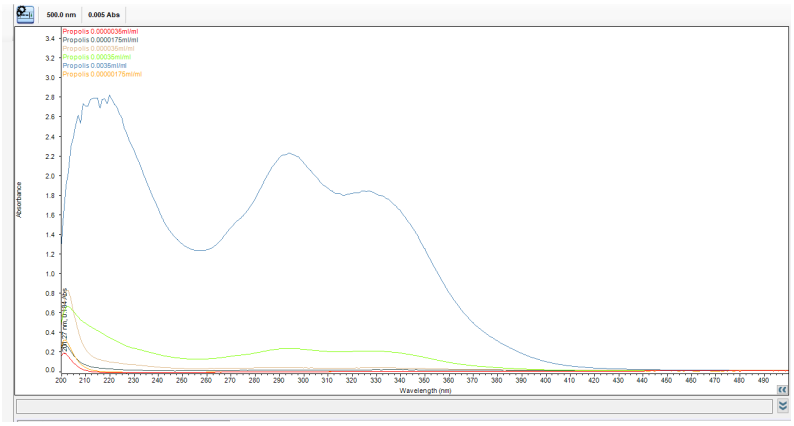


Figure 37: Spectral scan of propolis at a range of concentrations; 0.0175 ng/ml, 0.175 ng/ml, 0.35 ng/ml,1.75 ng/ml, 3.5 ng/ml, and 35 ng/ml. A scan of each concentration was done between 200nm and 500nm using the UV-vis spectrometer.

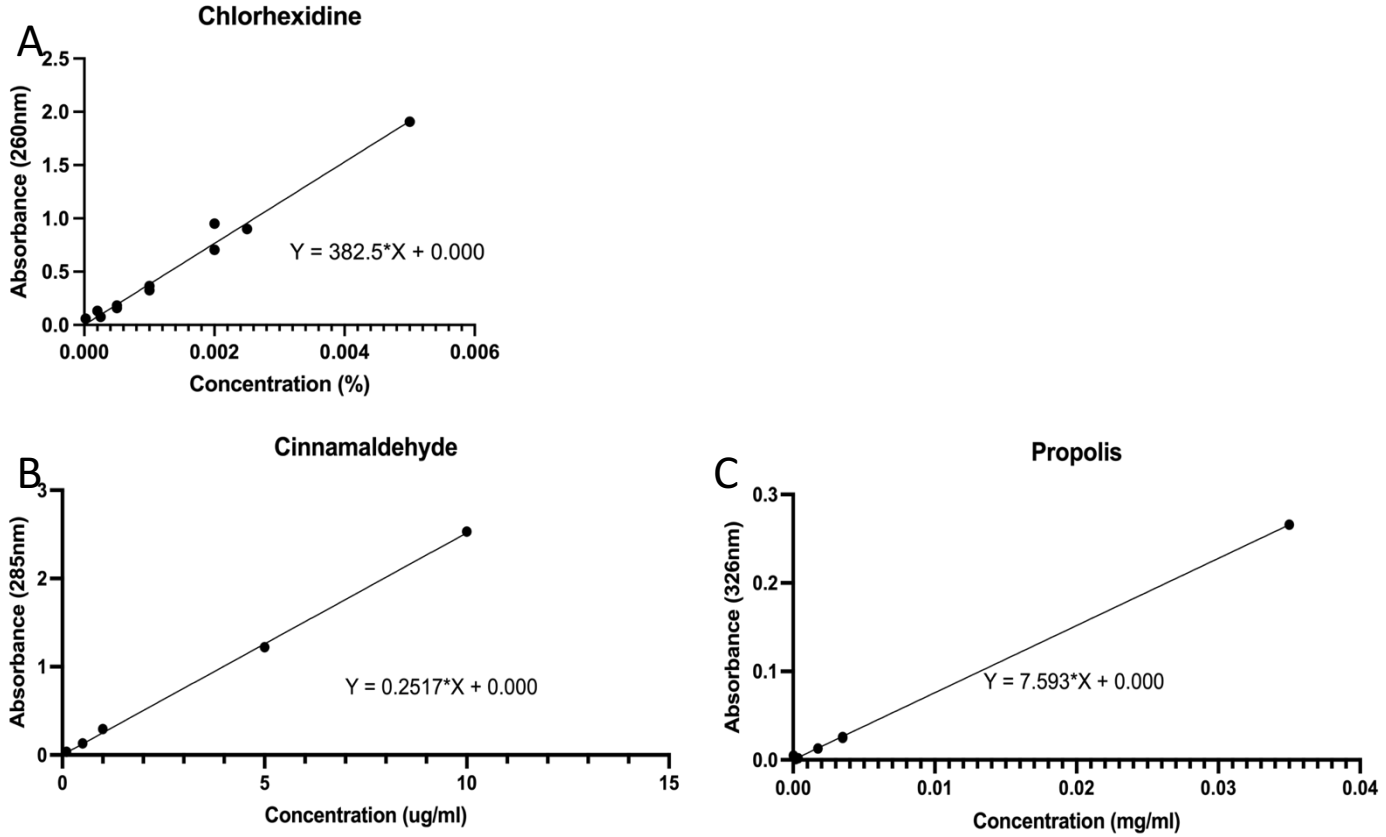


Figure 38: Linear regression graphs for chlorhexidine, cinnamaldehyde and propolis, using the UV-vis spectrometer.

Appendix E - Volunteer eligibility criteria for saliva and plaque collection.

Inclusion criteria:

- Participants must be over 18.
- Participants must maintain normal regular oral hygiene.
- Provide written informed consent to participate.

Exclusion criteria:

- Participants must be non-smokers.
- Participants must not have taken antibiotics in the last 3 months.
- Participants must not have been treated for active caries or periodontal disease.
- Participants must not be diabetic.
- Participants must not have long term use of anti-inflammatory medication.
- Participants must not have used an anti-inflammatory medication within the past 24 hours.
- Participants must not have symptoms of cold/flu.
- Participants must not be pregnant; think they may be pregnant or breastfeeding.
- Participants must not have history of jaw joint dysfunction (e.g. pain or clicking).
- Participants must not be full or partial dentures wearers.
- Participants must not be in current orthodontic treatment.
- Participants must not have oral piercings.
- Participants must not be Mondelez employees or relatives of thereof.
- Participants must not be students in the university department where this study is being performed.

Appendix F - Individual reactor exploration

a. Live stained bacteria

Figure 39 shows the percentage of live-stained bacteria for each reactor. In reactor 1, two treatments had a significant difference from the control; chewed with cinnamaldehyde and chewed with propolis. Both show a significant decrease in the percentage of live stained bacteria present, P=0.0025 and P=0.0006, respectively. In reactor 2, three treatments caused a significant increase in the percentage of live stained bacteria present; cinnamaldehyde (P=0.0191) propolis (P=0.0005) and chewed with cinnamaldehyde (P=0.0017).

Reactor 3 shows a significant reduction in the percentage of live stained bacteria present for all treatments except for chlorhexidine. Chlorhexidine alone caused no effect on the percentage of live stained bacteria present (P=0.8187). Whilst cinnamaldehyde and propolis caused a significant reduction (P=0.044 and P=0.011, respectively). Chewing also caused a significant reduction (P=0.0173), whilst the addition of cinnamaldehyde (P=0.0001), propolis (P=0.007) and a chlorhexidine (P=0.0073) to the chewing continued to cause a significant decrease from the control, they were not significantly different from the chewed control (P=0.3604, P=0.9851 and P=0.9982 respectively). These ANOVA values are presented in Table 11.

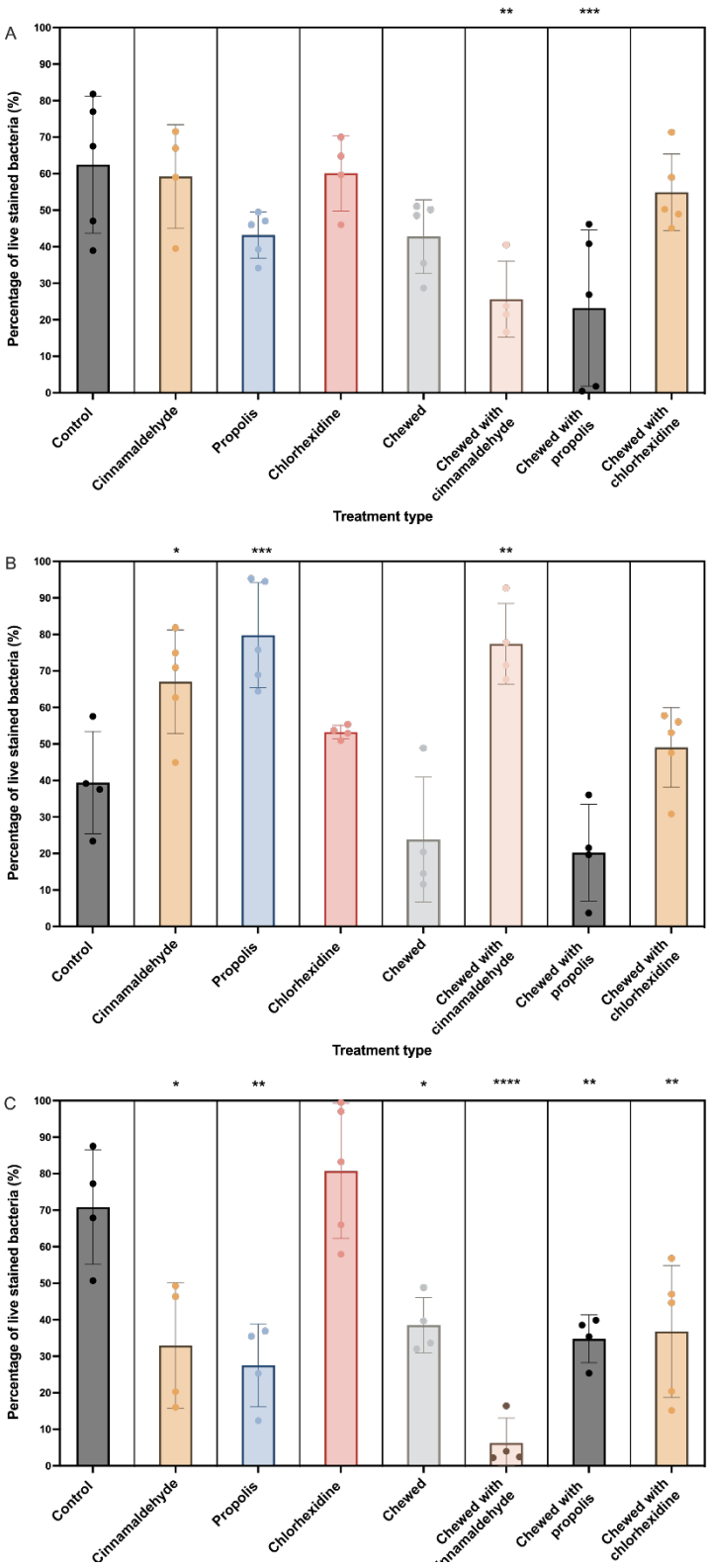


Figure 39: Graphs (A, B and C) showing the percentage of live stained bacteria for the individual reactors, A is data from reactor 1, B from Reactor 2, and C is from reactor 3. The IMARIS LIVE/DEAD image analysis of confocal laser scanning microscopy images is plotted. *Ex vivo* biofilms were grown for 5-days in a centre for disease control bioreactor in brain heart infusion supplemented with hemin, vitamin K and hog gastric mucin. These biofilms were then exposed to treatments for 15-minutes, these were chlorhexidine (0.2%), cinnamaldehyde (1000 g/ml), or propolis (0.035 g/ml), or exposed to manual chewing using the modified Wessel method, with and without chlorhexidine (0.2%), cinnamaldehyde (1000 g/ml), or propolis (0.035 g/ml). These biofilms were then stained with LIVE/DEAD stain and imaged on a confocal laser scanning microscopy. The biofilm z-stacks were then analysed in IMARIS to produce percentages of green-stained bacteria compared to red-stained bacteria. Mean ± 1 standard deviation, n=5. * = significance of difference from the control. *= p=0.05, **= p=0.01, ***= p=0.001, ****= p=0.0001.

Table 11: Analysis of variance of reactor treated samples compared to the control sample for the percentage of live stained bacteria from each reactor. The significantly different results were highlighted in red if the result was a significant decrease or in blue if it was a significant increase. Analysis of variance was calculated using GraphPad prism 9.

Treatment	Reactor 1	Reactor 2	Reactor 3
Cinnamaldehyde	0.9995	0.0191	0.0044
Propolis	0.1646	0.0005	0.0011
Chlorhexidine	0.9996	0.4976	0.8187
Chewed	0.1505	0.3781	0.0173
Chewed with cinnamaldehyde	0.0025	0.0017	<0.0001
Chewed with propolis	0.0006	0.1949	0.0070
Chewed with chlorhexidine	0.9227	0.7744	0.0073

b. Maximum biofilm thickness

In reactor 1, the biofilm thickness varied between 10 µm to 80 µm, but none of the treatments affected the maximum biofilm thickness. In reactor 2, a significant increase was observed when the *ex vivo* biofilms were exposed to chlorhexidine (P=0.0001), and a significant decrease in biofilm thickness was observed when exposed to chewing in the presence of cinnamaldehyde (P=0.0007). In contrast, reactor 3 had two treatments which caused an increase in biofilm maximum thickness; propolis exposure (P=0.0301) and chewed in the presence of chlorhexidine (P=0.0001). Chewed with chlorhexidine in reactor 3 caused the largest increase of any reactor, with a maximum biofilm thickness of 150 µm.

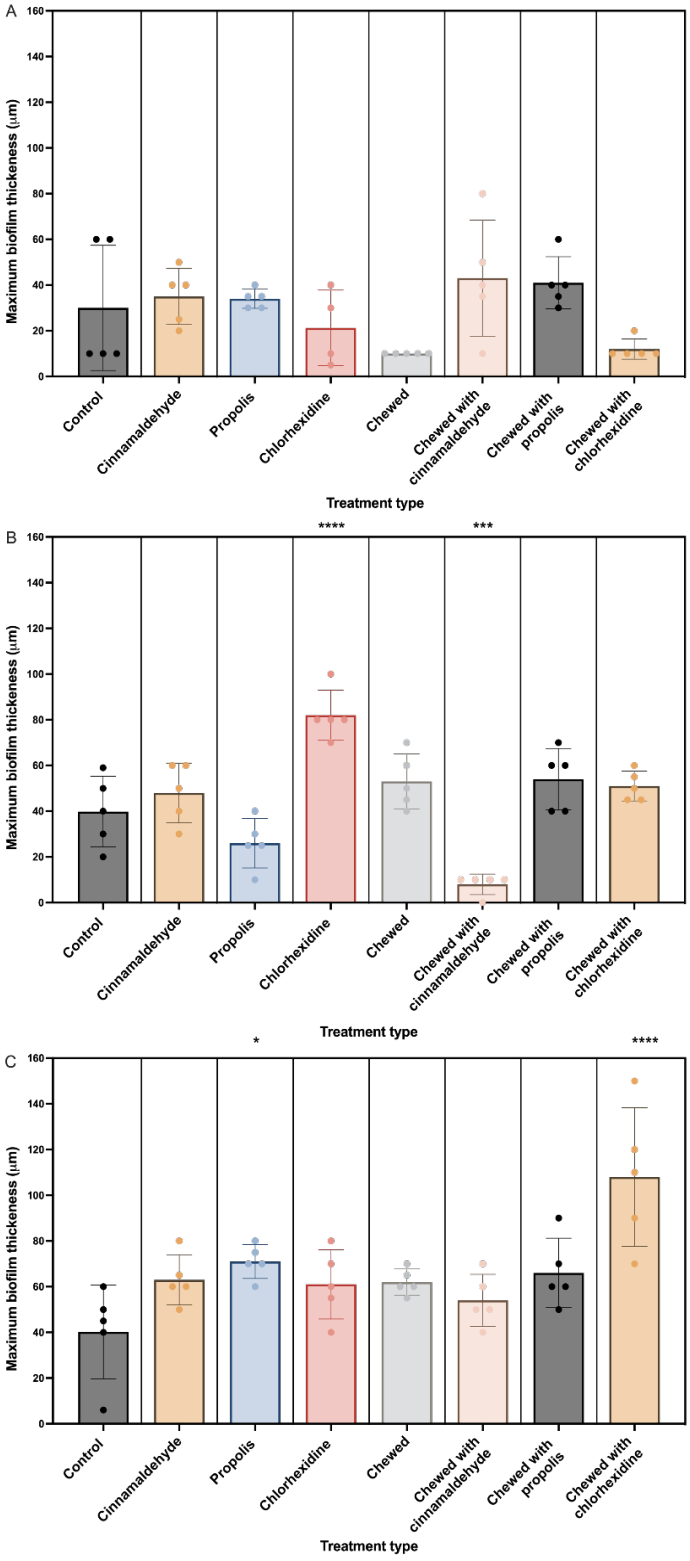


Figure 40: Graphs (A, B and C) showing maximum biofilm thickness for the individual reactors, A is data from reactor 1, B from Reactor 2, and C is from reactor 3. IMARIS image analysis of confocal laser scanning microscopy images. *Ex vivo* biofilms were grown for 5-days in a Centre for Disease Control bioreactor in brain heart infusion supplemented with hemin, vitamin K and hog gastric mucin. These biofilms were then exposed to treatments for 15-minutes, these were chlorhexidine (0.2%), cinnamaldehyde (1000 g/ml), or propolis (0.035 g/ml), or exposed to manual chewing using the modified Wessel method, with and without chlorhexidine (0.2%), cinnamaldehyde (1000 g/ml), or propolis (0.035 g/ml). These biofilms were then stained with LIVE/DEAD stain and imaged on a confocal laser scanning microscopy. The biofilm z-stacks were then analysed in IMARIS to produce biofilm thickness data. Mean ± 1 standard deviation, n=5. * = significance of difference from the control. *= p=0.05, **= p=0.01, ***= p=0.001, ****= p=0.0001.

Table 12: Analysis of variance of reactor treated samples compared to the control sample for the maximum biofilm thickness from each reactor. The significantly different results were highlighted in red if the result was a significant decrease or in blue if it was a significant increase. Analysis of variance was calculated using GraphPad prism 9.

Treatment	Reactor 1	Reactor 2	Reactor 3
Cinnamaldehyde	0.9946	0.7692	0.1621
Propolis	0.9979	0.2738	0.0301
Chlorhexidine	0.9345	<0.0001	0.2325
Chewed	0.2356	0.3150	0.1948
Chewed with cinnamaldehyde	0.6577	0.0007	0.6340
Chewed with propolis	0.7944	0.2486	0.0899
Chewed with chlorhexidine	0.3316	0.4800	<0.0001

c. Percentage of disc coverage

Figure 41A indicates that there was no significant difference in percentage coverage of the disc across any of the treatments of the *ex vivo* biofilms in reactor 1. However, there was a large amount of variation across each sample, with the control having a range from 4% to 100%. Over the five images from the chewed with chlorhexidine disc, the results spanned from 0% to 100% coverage on the same disc, with the chewed disc showing nearly complete removal. The geometric means of each treatment varied from the control at 31% to propolis exposure at 65% and down to 2% for the disc exposed to chewing; however, due to the wide differences within the samples, none of these differences were statistically significant.

In reactor 2, the percentage coverage of the disc remained stable across all the samples, with some variation across the discs. Chewed with cinnamaldehyde was the only sample to be significantly different from the control, with a near complete removal from the disc. In contrast to this, the chewed disc was observed to be 100% covered in all five images. In reactor 3, there were minimal changes to the percentage coverage of the disc, with 38 of the 40 images having a coverage of over 90%. Across the three reactors, only one value, chewed with cinnamaldehyde in reactor 2, was significantly different from the control, as seen in Table 13.

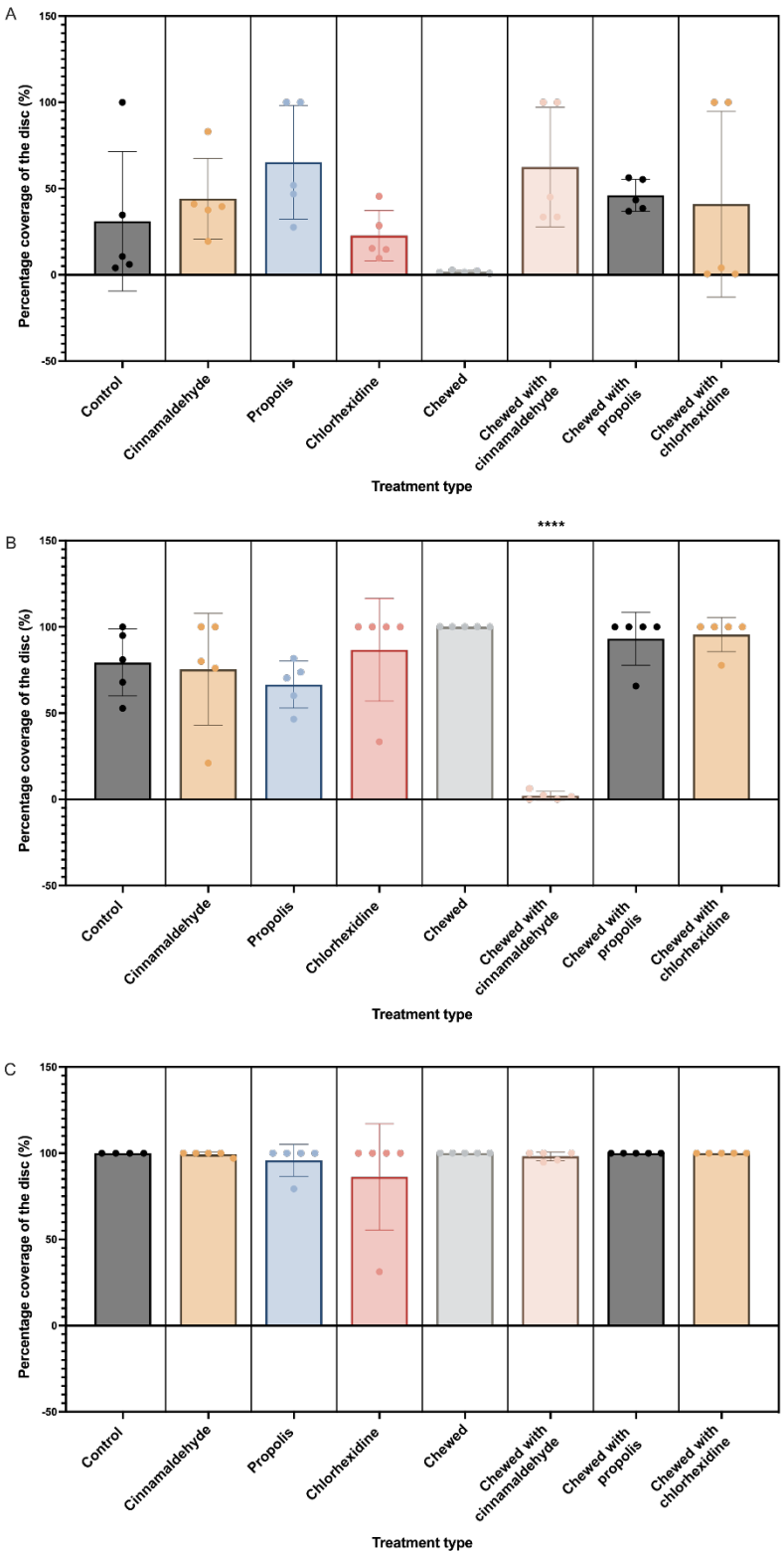


Figure 41: Graphs (A, B and C) showing percentage coverage of the disc for the individual reactors, A is data from reactor 1, B from Reactor 2, and C is from reactor 3. Fuji image analysis of confocal laser scanning microscopy images. *Ex vivo* biofilms were grown for 5-days in a Centre for Disease Control bioreactor in brain heart infusion supplemented with hemin, vitamin K and hog gastric mucin. These biofilms were then exposed to treatments for 15-minutes, these were chlorhexidine (0.2%), cinnamaldehyde (1000 g/ml), or propolis (0.035 g/ml), or exposed to manual chewing using the modified Wessel method, with and without chlorhexidine (0.2%), cinnamaldehyde (1000 g/ml), or propolis (0.035 g/ml). These biofilms were then stained with LIVE/DEAD stain and imaged on a confocal laser scanning microscopy. Mean ± 1 standard deviation, n=5. * = significance of difference from the control. *= p=0.05, **= p=0.01, ***= p=0.001, ****= p=0.0001.

Table 13: Analysis of variance of reactor treated samples compared to the control sample for the percentage of disc coverage from each reactor. The significantly different results were highlighted in red if the result was a significant decrease or in blue if it was a significant increase. Analysis of variance was calculated using GraphPad prism 9.

Treatment	Reactor 1	Reactor 2	Reactor 3
Cinnamaldehyde	0.9769	0.9995	0.9999
Propolis	0.3631	0.8089	0.9918
Chlorhexidine	0.9976	0.9850	0.3312
Chewed	0.5200	0.3699	>0.9999
Chewed with cinnamaldehyde	0.4502	<0.0001	0.9996
Chewed with propolis	0.9538	0.7570	>0.9999
Chewed with chlorhexidine	0.9943	0.6162	>0.9999

Bibliography

A. Yamanaka R. Kimizuka T. Kato K. Okuda (2004) 'Inhibitory effects of cranberry juice on attachment of oral streptococci and biofilm formation', *Oral Microbiology and Immunology*, 19(3).

Aas, J. A. *et al.* (2005) 'Defining the normal bacterial flora of the oral cavity.', *Journal of clinical microbiology*, 43(11), pp. 5721–32. doi: 10.1128/JCM.43.11.5721-5732.2005.

Abbafati, C. *et al.* (2020) 'Global burden of 369 diseases and injuries in 204 countries and territories, 1990–2019: a systematic analysis for the Global Burden of Disease Study 2019', *The Lancet*, 396(10258), pp. 1204–1222. doi: 10.1016/S0140-6736(20)30925-9.

Abranches, J. *et al.* (2018) 'Biology of Oral Streptococci', *Microbiology Spectrum*, 6(5). doi: 10.1128/microbiolspec.gpp3-0042-2018.

Acinas, S. G. *et al.* (2005) 'PCR-induced sequence artifacts and bias: Insights from comparison of two 16s rRNA clone libraries constructed from the same sample', *Applied and Environmental Microbiology*, 71(12), pp. 8966–8969. doi: 10.1128/AEM.71.12.8966-8969.2005.

Ahn, J. *et al.* (2011) 'Oral microbiome profiles: 16s rRNA pyrosequencing and microarray assay comparison', *PLoS ONE*, 6(7), pp. 1–6. doi: 10.1371/journal.pone.0022788.

Aksoy, A., Duran, N. and Koksal, F. (2006) 'In vitro and in vivo antimicrobial effects of mastic chewing gum against Streptococcus mutans and mutans streptococci', *Archives of Oral Biology*, 51(6), pp. 476–481. doi: 10.1016/J.ARCHORALBIO.2005.11.003.

Al-Ahmad, A. *et al.* (2010) 'Biofilm formation and composition on different implant materials in vivo', *Journal of Biomedical Materials Research - Part B Applied Biomaterials*, 95(1), pp. 101–109. doi: 10.1002/jbm.b.31688.

Al-Kamel, A. *et al.* (2019) 'Subgingival microbiome of experimental gingivitis: shifts associated with the use of chlorhexidine and N-acetyl cysteine mouthwashes', *Journal of Oral Microbiology*, 11(1). doi: 10.1080/20002297.2019.1608141.

An, S. Q. *et al.* (2022) 'An in vitro biofilm model system to facilitate study of microbial communities of the human oral cavity', *Letters in Applied Microbiology*, 74(3), pp. 302–310. doi: 10.1111/lam.13618.

Azeredo, J. *et al.* (2017) 'Critical review on biofilm methods', *Critical Reviews in Microbiology*,

43(3), pp. 313–351. doi: 10.1080/1040841X.2016.1208146.

Baehren, C. *et al.* (2023) 'The Overlooked Microbiome—Considering Archaea and Eukaryotes Using Multiplex Nanopore-16S-/18S-rDNA-Sequencing: A Technical Report Focusing on Nasopharyngeal Microbiomes', *International Journal of Molecular Sciences*, 24(2), pp. 1–12. doi: 10.3390/ijms24021426.

Bakker, D. P. *et al.* (2004) 'Bacterial strains isolated from different niches can exhibit different patterns of adhesion to substrata', *Applied and Environmental Microbiology*, 70(6), pp. 3758–3760. doi: 10.1128/AEM.70.6.3758-3760.2004.

Balasubramanian, A. R. *et al.* (2021) 'Combinatorial effects of trans-cinnamaldehyde with fluoride and chlorhexidine on Streptococcus mutans', *Journal of Applied Microbiology*, 130(2), pp. 382–393. doi: 10.1111/jam.14794.

Banasb, J. A. (2015) 'Codon-optimized Fluorescent mTFP and mCherry for Microscopic Visualization and Genetic Counterselection of Streptococci and Enterococci', *Microbiology Methods*, 116(1), pp. 15–22. doi: 10.1016/j.mimet.2015.06.010.Codon-optimized.

Barraud, N. *et al.* (2014) 'Nitric Oxide: A Key Mediator of Biofilm Dispersal with Applications in Infectious Diseases', *Current Pharmaceutical Design*, 21(1), pp. 31–42. doi: 10.2174/13816128206661409051128 22.

Bartold, P. M. and Van Dyke, T. E. (2019) 'An appraisal of the role of specific bacteria in the initial pathogenesis of periodontitis', *Journal of Clinical Periodontology*, 46(1), pp. 6–11. doi: 10.1111/jcpe.13046.

Beck, J. D. *et al.* (2019) 'Periodontal Medicine: 100 Years of Progress', *Journal of Dental Research*, 98(10), pp. 1053–1062. doi: 10.1177/0022034519846113.

Becker, K. *et al.* (2021) 'Efficacy of 0.05% chlorhexidine and 0.05% cetylpyridinium chloride mouthwash to eliminate living bacteria on in situ collected biofilms: An in vitro study', *Antibiotics*, 10(6). doi: 10.3390/antibiotics10060730.

Belay, A. (2012) 'Self-association , sodium ion complexation and optical transition probabilities of caffeic acid determined spectrophotometrically', *Journal of Biological Physics and Chemistry*, 12(November 2011), pp. 23–28.

Benachinmardi, K. K. *et al.* (2015) 'Microbial Flora in Chronic Periodontitis: Study at a Tertiary Health Care Center from North Karnataka', *Journal of Laboratory Physicians*, 7(01), pp. 049–054.

doi: 10.4103/0974-2727.154798.

Besnard, V. *et al.* (2002) 'Environmental and physico-chemical factors induce VBNC state in Listeria monocytogenes', *Veterinary Research*, 33, pp. 359–370. doi: 10.1051/vetres.

Bik, E. M. *et al.* (2010) 'Bacterial diversity in the oral cavity of 10 healthy individuals', *The ISME Journal*, 4(8), pp. 962–974. doi: 10.1038/ismej.2010.30.

van der Bilt, A. and Abbink, J. H. (2017) 'The influence of food consistency on chewing rate and muscular work', *Archives of Oral Biology*, 83(June), pp. 105–110. doi: 10.1016/j.archoralbio.2017.07.011.

BiosurfaceTechnologies (2019) *Bio-inLine Biofilm Reactor - BioSurface Technologies*. Available at: https://biofilms.biz/products/biofilm-reactors/bio-inline-biofilm-reactor/ (Accessed: 30 May 2019).

Bloch, S. *et al.* (2017) 'Behavior of two Tannerella forsythia strains and their cell surface mutants in multispecies oral biofilms', *Molecular Oral Microbiology*, 32(5), pp. 404–418. doi: 10.1111/omi.12182.

Bos, R., van der Mei, H. C. and Busscher, H. J. (1999) 'Physico-chemistry of initial microbial adhesive interactions – its mechanisms and methods for study', *FEMS Microbiology Reviews*, 23(2), pp. 179–230. doi: 10.1111/j.1574-6976.1999.tb00396.x.

Botelho, J. *et al.* (2022) 'An umbrella review of the evidence linking oral health and systemic noncommunicable diseases', *Nature Communications*, 13(1). doi: 10.1038/s41467-022-35337-8.

Bowen, W. H. and Koo, H. (2011) 'Biology of streptococcus mutans-derived glucosyltransferases: Role in extracellular matrix formation of cariogenic biofilms', *Caries Research*, pp. 69–86. doi: 10.1159/000324598.

Brakhage, A. (2005) 'Systemic Fungal Infections Caused by Aspergillus Species: Epidemiology, Infection Process and Virulence Determinants', *Current Drug Targets*, 6(8), pp. 875–886. doi: 10.2174/138945005774912717.

Brookes, Zo¨e L.S. ,Bescos, R. (2020) 'Current uses of chlorhexidine for management of oral disease: a narrative review', *Journal of Dentistry*, 103(January), p. 293.

Brooks, J. P. *et al.* (2015) 'The truth about metagenomics: Quantifying and counteracting bias in 16S rRNA studies Ecological and evolutionary microbiology', *BMC Microbiology*, 15(1), pp. 1–14. doi: 10.1186/s12866-015-0351-6.

Brown, J. L. *et al.* (2019) 'Polymicrobial oral biofilm models: Simplifying the complex', *Journal of Medical Microbiology*, 68(11), pp. 1573–1584. doi: 10.1099/JMM.0.001063.

Carlsson, J., Iwami, Y. and Yamada, T. (1983) 'Hydrogen peroxide excretion by oral streptococci and effect of lactoperoxidase-thiocyanate-hydrogen peroxide', *Infection and Immunity*, 40(1), pp. 70–80. doi: 10.1128/iai.40.1.70-80.1983.

Caselli, E. *et al.* (2020) 'Defining the oral microbiome by whole-genome sequencing and resistome analysis: The complexity of the healthy picture', *BMC Microbiology*, 20(1), pp. 1–19. doi: 10.1186/s12866-020-01801-y.

Ceri, H. *et al.* (1999) 'The Calgary Biofilm Device: New Technology for Rapid Determination of Antibiotic Susceptibilities of Bacterial Biofilms', *Journal of Clinical Microbiology*, 37(6), pp. 1771–1776.

Chen, D.-Y. *et al.* (2016) 'Risk of Atrial Fibrillation or Flutter Associated with Periodontitis: A Nationwide, Population-Based, Cohort Study.', *PloS one*, 11(10), p. e0165601. doi: 10.1371/journal.pone.0165601.

Cheng, S. *et al.* (2023) 'Citral and trans-cinnamaldehyde, two plant-derived antimicrobial agents can induce Staphylococcus aureus into VBNC state with different characteristics', *Food Microbiology*, 112(November 2022), p. 104241. doi: 10.1016/j.fm.2023.104241.

Cherukuri, S. R. (1987) 'Stable cinnamon-flavored chewing gum composition.', *European patent office*.

Chewing Gum Tester DRT - ERWEKA GmbH (2019). Available at: https://www.erweka.com/products/dissolution-testers/chewing-gum-tester.html (Accessed: 30 May 2019).

Chug, M. K. *et al.* (2022) 'Prevention of medical device infections via multi-action nitric oxide and chlorhexidine diacetate releasing medical grade silicone biointerfaces', *Journal of Biomedical Materials Research - Part A*, 110(6), pp. 1263–1277. doi: 10.1002/jbm.a.37372.

Chye, R. M. L. *et al.* (2019) 'Effectiveness of Different Commercial Chlorhexidine-Based Mouthwashes after Periodontal and Implant Surgery: A Systematic Review', *Implant Dentistry*, 28(1), pp. 74–85. doi: 10.1097/ID.0000000000000854.

Cornforth, D. M. *et al.* (2014) 'Combinatorial quorum sensing allows bacteria to resolve their social and physical environment', *Proceedings of the National Academy of Sciences of the United*

States of America, 111(11), pp. 4280–4284. doi: 10.1073/pnas.1319175111.

Crusz, S. A. *et al.* (2012) 'Bursting the bubble on bacterial biofilms: A flow cell methodology', *Biofouling*, 28(8), pp. 835–842. doi: 10.1080/08927014.2012.716044.

Cushnie, T. P. T. and Lamb, A. J. (2005) 'Detection of galangin-induced cytoplasmic membrane damage in Staphylococcus aureus by measuring potassium loss', *Journal of Ethnopharmacology*, 101(1–3), pp. 243–248. doi: 10.1016/j.jep.2005.04.014.

Dale, B. A. *et al.* (2006) 'Oral antimicrobial peptides and biological control of caries', *BMC Oral Health*, 6(SUPPL. 1), pp. 1–7. doi: 10.1186/1472-6831-6-S1-S13.

Darrene, L.-N. and Cecile, B. (2016) 'Experimental Models of Oral Biofilms Developed on Inert Substrates: A Review of the Literature.', *BioMed research international*, 2016, p. 7461047. doi: 10.1155/2016/7461047.

Deborah M Lyle, C Ram Goyal, Jimmy G Qaqish, R. S. (2016) 'Comparison of Water Flosser and Interdental Brush on Plaque Removal: A Single-Use Pilot Study', *Journal of Clinical Dentistry*, (27), pp. 23–26.

DeJong, A. E. and Hartel, R. W. (2020) 'Quantification of γ-sorbitol crystal growth rate and solubility in the presence of mannitol and maltitol', *Journal of Food Science*, 85(12), pp. 4319–4326. doi: 10.1111/1750-3841.15519.

DeJong, A. E. and Hartel, R. W. (2021) 'Modulating sorbitol crystallization using impurities', *Journal of Food Engineering*, 296(December 2020), p. 110475. doi: 10.1016/j.jfoodeng.2020.110475.

Devienne, G. *et al.* (2018) 'Single cell multiplex reverse transcription polymerase chain reaction after patch-clamp', *Journal of Visualized Experiments*, 2018(136), pp. 1–12. doi: 10.3791/57627.

Dewhirst, F. E. *et al.* (2010) 'The human oral microbiome.', *Journal of bacteriology*, 192(19), pp. 5002–17. doi: 10.1128/JB.00542-10.

Dhadse, P., Gattani, D. and Mishra, R. (2010) 'The link between periodontal disease and cardiovascular disease: How far we have come in last two decades ?', *Journal of Indian Society of Periodontology*, 14(3), pp. 148–54. doi: 10.4103/0972-124X.75908.

Dige, I. *et al.* (2009) 'Application of stereological principles for quantification of bacteria in intact dental biofilms', *Oral Microbiology and Immunology*, 24(1), pp. 69–75. doi: 10.1111/j.1399-302X.2008.00482.x.

Domadia, P. *et al.* (2007) 'Inhibition of bacterial cell division protein FtsZ by cinnamaldehyde', *Biochemical Pharmacology*, 74(6), pp. 831–840. doi: 10.1016/j.bcp.2007.06.029.

Dominy, S. S. *et al.* (2019) '*Porphyromonas gingivalis* in Alzheimer's disease brains: Evidence for disease causation and treatment with small-molecule inhibitors', *Science Advances*, 5(1), p. eaau3333. doi: 10.1126/sciadv.aau3333.

Drescher, K. *et al.* (2014) 'Solutions to the public goods dilemma in bacterial biofilms', *Current Biology*, 24(1), pp. 50–55. doi: 10.1016/j.cub.2013.10.030.

Drugs.com (2019) *Trident White Sugarless Gum Information from*. Available at: https://www.drugs.com/drp/trident-white-sugarless-gum.html (Accessed: 30 May 2019).

Du, Q. *et al.* (2020) 'Sucrose promotes caries progression by disrupting the microecological balance in oral biofilms: an in vitro study', *Scientific Reports*, 10(1), pp. 1–12. doi: 10.1038/s41598-020-59733-6.

Duizer, L. M., Bloom, K. and Findlay, C. J. (1996) 'Dual-attribute time-intensity measurement of sweetness and peppermint perception of chewing gum', *Journal of Food Science*, 61(3), pp. 636–638. doi: 10.1111/j.1365-2621.1996.tb13175.x.

Edlund, A. *et al.* (2013) 'An in vitro biofilm model system maintaining a highly reproducible species and metabolic diversity approaching that of the human oral microbiome', *Microbiome*, 1(1), pp. 1–17. doi: 10.1186/2049-2618-1-25.

Edlund, A. *et al.* (2017) 'Metabolic Fingerprints from the Human Oral Microbiome Reveal a Vast Knowledge Gap of Secreted Small Peptidic Molecules.', *mSystems*, 2(4). doi: 10.1128/mSystems.00058-17.

Fanning, S. and Mitchell, A. P. (2012) 'Fungal biofilms.', *PLoS pathogens*, 8(4), pp. 1–4. doi: 10.1371/journal.ppat.1002585.

Featherstone, J. (2008) 'Dental caries: a dynamic disease process', *Australian Dental Journal*, 53(3), pp. 286–291. doi: 10.1111/j.1834-7819.2008.00064.x.

Fernández, C. E. *et al.* (2017) 'The effect of inoculum source and fluid shear force on the development of in vitro oral multispecies biofilms', *Journal of Applied Microbiology*, 122(3), pp. 796–808. doi: 10.1111/jam.13376.

Figuero, E., Han, Y. W. and Furuichi, Y. (2020) 'Periodontal diseases and adverse pregnancy outcomes: Mechanisms', *Periodontology 2000*, 83(1), pp. 175–188. doi: 10.1111/prd.12295.

Firmino, D. F. *et al.* (2018) 'Antibacterial and Antibiofilm Activities of Cinnamomum Sp. Essential Oil and Cinnamaldehyde: Antimicrobial Activities', *Scientific World Journal*, 2018. doi: 10.1155/2018/7405736.

García-Salinas, S. *et al.* (2018) 'Evaluation of the antimicrobial activity and cytotoxicity of different components of natural origin present in essential oils', *Molecules*, 23(6), pp. 1–18. doi: 10.3390/molecules23061399.

Gebara, E. C. E., Lima, L. A. and Mayer, M. P. A. (2002) 'Propolis antimicrobial activity against periodontopathic bacteria', *Brazilian Journal of Microbiology*, 33(4), pp. 365–369. doi: 10.1590/S1517-83822002000400018.

GenoHub (2019) *Whole Genome Sequencing and Re-sequencing Guide*.

Ghannoum, M. A. *et al.* (2010) 'Characterization of the oral fungal microbiome (mycobiome) in healthy individuals', *PLoS Pathogens*, 6(1). doi: 10.1371/journal.ppat.1000713.

Gill, A. O. and Holley, R. A. (2004) 'Mechanisms of bactericidal action of cinnamaldehyde against Listeria monocytogenes and of eugenol against L. monocytogenes and Lactobacillus sakei', *Applied and Environmental Microbiology*, 70(10), pp. 5750–5755. doi: 10.1128/AEM.70.10.5750-5755.2004.

Goldenberg, R. L. and Culhane, J. F. (2007) 'Low birth weight in the United States', *The American Journal of Clinical Nutrition*, 85(2), pp. 584S-590S. doi: 10.1093/ajcn/85.2.584S.

Górniak, I., Bartoszewski, R. and Króliczewski, J. (2019) *Comprehensive review of antimicrobial activities of plant flavonoids*, *Phytochemistry Reviews*. doi: 10.1007/s11101-018-9591-z.

Gozalbo, D. *et al.* (2004) 'Candida and candidiasis: The cell wall as a potential molecular target for antifungal therapy', *Current Drug Targets - Infectious Disorders*, 4(2), pp. 117–135. doi: 10.2174/1568005043341046.

Gross, E. L. *et al.* (2012) 'Beyond Streptococcus mutans: Dental Caries Onset Linked to Multiple Species by 16S rRNA Community Analysis', *PLoS ONE*, 7(10). doi: 10.1371/journal.pone.0047722.

Grover, H. S. *et al.* (2015) 'Detection and measurement of oral malodor in chronic periodontitis patients and its correlation with levels of select oral anaerobes in subgingival plaque', *Contemporary Clinical Dentistry*, 6, pp. S181–S187. doi: 10.4103/0976-237X.166825.

Guggenheim, B. *et al.* (2001) *Validation of an in vitro Biofilm Model of Supragingival Plaque*, *J Dent Res*. Available at: http://journals.sagepub.com/doi/pdf/10.1177/00220345010800011201

(Accessed: 29 October 2018).

Guggenheim, B. *et al.* (2004) 'Application of the Zürich Biofilm Model to Problems of Cariology'. doi: 10.1159/000077757.

Guo, W. *et al.* (2015) 'Determining the resistance of carbapenemresistant Klebsiella pneumoniae to common disinfectants and elucidating the underlying resistance mechanisms', *Pathogens and Global Health*, 109(4), pp. 184–192. doi: 10.1179/2047773215Y.0000000022.

Hajishengallis, G., Darveau, R. P. and Curtis, M. A. (2012) 'The keystone-pathogen hypothesis.', *Nature reviews. Microbiology*, 10(10), pp. 717–25. doi: 10.1038/nrmicro2873.

Haney, E. F. *et al.* (2018) 'Critical assessment of methods to quantify biofilm growth and evaluate antibiofilm activity of host defence peptides', *Biomolecules*, 8(2), pp. 1–22. doi: 10.3390/biom8020029.

Hannig, M. (1999) 'Ultrastructural investigation of pellicle morphogenesis at two different intraoral sites during a 24-h period.', *Clinical oral investigations*, 3(2), pp. 88–95. doi: 10.1007/s007840050084.

Hannig, M. and Joiner, A. (2006) 'The structure, function and properties of the acquired pellicle', in *The Teeth and Their Environment: Physical, Chemical and Biochemical Influences*, pp. 29–64.

He, Z. *et al.* (2019) 'Antimicrobial Activity of Cinnamaldehyde on Streptococcus mutans Biofilms', *Frontiers in Microbiology*, 10(September), pp. 1–11. doi: 10.3389/fmicb.2019.02241.

Hendrickx, L., Hausner, M. and Wuertz, S. (2003) 'Natural genetic transformation in monoculture Acinetobacter sp. strain BD413 biofilms', *Applied and Environmental Microbiology*, 69(3), pp. 1721–1727. doi: 10.1128/AEM.69.3.1721-1727.2003.

Hennessey, T. D. (1973) 'Some antibacterial properties of chlorhexidine', *Journal of Periodontal Research*, pp. 61–67. doi: 10.1111/j.1600-0765.1973.tb02166.x.

Hermansson, M. (1999) 'The DLVO theory in microbial adhesion', *Colloids and Surfaces B: Biointerfaces*, 14(1–4), pp. 105–119. doi: 10.1016/S0927-7765(99)00029-6.

Hospenthal, M. K., Costa, T. R. D. and Waksman, G. (2017) 'A comprehensive guide to pilus biogenesis in Gram-negative bacteria', *Nature Reviews Microbiology*, 15(6), pp. 365–379. doi: 10.1038/nrmicro.2017.40.

How, K. Y., Song, K. P. and Chan, K. G. (2016) 'Porphyromonas gingivalis: An Overview of

Periodontopathic Pathogen below the Gum Line.', *Frontiers in microbiology*, 7, p. 53. doi: 10.3389/fmicb.2016.00053.

Huang, P. *et al.* (2021) 'Efficacy of Er:YAG laser irradiation for decontamination and its effect on biocompatibility of different titanium surfaces', *BMC Oral Health*, 21(1), pp. 1–12. doi: 10.1186/s12903-021-02006-z.

Hube, B. (2004) 'From commensal to pathogen: Stage- and tissue-specific gene expression of Candida albicans', *Current Opinion in Microbiology*, 7(4), pp. 336–341. doi: 10.1016/j.mib.2004.06.003.

Huebinger, J. (2018) 'Modification of cellular membranes conveys cryoprotection to cells during rapid, nonequilibrium cryopreservation', *PLoS ONE*, 13(10), pp. 1–13. doi: 10.1371/journal.pone.0205520.

Humphrey, S. P. R. T. W. (2001) *A review of saliva: Normal composition, flow, and function*. Available at: https://www.thejpd.org/article/S0022-3913(01)54032-9/pdf (Accessed: 29 April 2019).

Imfeld, T. (2006) 'Chlorhexidine-containing chewing gum', *Zurich Open Repository and Archive*, 116, pp. 476–483.

Information, N. C. for B. (2023) *PubChem Cinnamaldehyde*. Available at: https://pubchem.ncbi.nlm.nih.gov/compound/Cinnamaldehyde.

Ioannou, C. J., Hanlon, G. W. and Denyer, S. P. (2007) 'Action of disinfectant quaternary ammonium compounds against Staphylococcus aureus', *Antimicrobial Agents and Chemotherapy*, 51(1), pp. 296–306. doi: 10.1128/AAC.00375-06.

Iversen, M. *et al.* (2007) 'Aspergillus infection in lung transplant patients: Incidence and prognosis', *European Journal of Clinical Microbiology and Infectious Diseases*, 26(12), pp. 879–886. doi: 10.1007/s10096-007-0376-3.

J W Wimpenny (1997) 'The validity of models', *SAGE*. doi: 0.1177/08959374970110010601.

Jacobsen, J., Christrup, L. L. and Jensen, N. H. (2004) 'Medicated chewing gum: Pros and cons', *American Journal of Drug Delivery*, 2(2), pp. 75–88. doi: 10.2165/00137696-200402020-00001.

Jenkins, S., Addy, M. and Wade, W. (1988) 'The mechanism of action of chlorhexidine: A study of plaque growth on enamel inserts in vivo', *Journal of Clinical Periodontology*, 15(7), pp. 415–424. doi: 10.1111/j.1600-051X.1988.tb01595.x.

Jukka Ainamo, H. E. (1987) 'Prevention of plaque growth with chewing gum containing chlorhexidine acetate', *Journal of Clinical Periodontology*, 14(9), pp. 524–527.

Jung, J. Y. *et al.* (2018) 'Rapid oral bacteria detection based on real-time PCR for the forensic identification of saliva', *Scientific Reports*, 8(1), pp. 2–11. doi: 10.1038/s41598-018-29264-2.

Kannan, M. *et al.* (2017) 'Silver Iodide Nanoparticles as an Antibiofilm Agent—A Case Study on Gram-Negative Biofilm-Forming Bacteria', *Nanostructures for Antimicrobial Therapy*, pp. 435–456. doi: 10.1016/B978-0-323-46152-8.00019-6.

Kaplan, J. B. (2010) 'Biofilm Dispersal: Mechanisms, Clinical Implications, and Potential Therapeutic Uses', *Journal of Dental Research*, 89(3), pp. 205–218. doi: 10.1177/0022034509359403.

Kara, D. *et al.* (2007) 'Microstructural differences between single-species and dual-species biofilms of Streptococcus mutans and Veillonella parvula, before and after exposure to chlorhexidine', *FEMS Microbiology Letters*, 271(1), pp. 90–97. doi: 10.1111/j.1574-6968.2007.00701.x.

Kawasaki, T., Takahashi, S. and Ideda, K. (1985) 'Hydroxyapatite high-performance liquid chromatography: column performance for proteins', *European Journal of Biochemistry*, 152(2), pp. 361–371. doi: 10.1111/j.1432-1033.1985.tb09206.x.

Kazor, C. E. *et al.* (2003) 'Diversity of bacterial populations on the tongue dorsa of patients with halitosis and healthy patients.', *Journal of clinical microbiology*, 41(2), pp. 558–63. doi: 10.1128/JCM.41.2.558-563.2003.

Keijser, B. J. F. *et al.* (2018) 'The Impact of Maltitol-Sweetened Chewing Gum on the Dental Plaque Biofilm Microbiota Composition', *Frontiers in Microbiology*, 9. doi: 10.3389/fmicb.2018.00381.

Kemp, B. M. and Smith, D. G. (2005) 'Use of bleach to eliminate contaminating DNA from the surface of bones and teeth', *Forensic Science International*, 154(1), pp. 53–61. doi: 10.1016/j.forsciint.2004.11.017.

Khairnar, M. R. *et al.* (2015) 'Comparative assessment of Cranberry and Chlorhexidine mouthwash on streptococcal colonization among dental students: A randomized parallel clinical trial', *Contemporary Clinical Dentistry*, 6(1), pp. 35–39. doi: 10.4103/0976-237X.149289.

Kharitonova, M. *et al.* (2021) 'The composition of microbial communities in inflammatory periodontal diseases in young adults tatars', *AIMS Microbiology*, 7(1), pp. 59–74. doi:

10.3934/microbiol.2021005.

Kim, H., Kim, H. W. and Jung, S. (2008) 'Aqueous solubility enhancement of some flavones by complexation with cyclodextrins', *Bulletin of the Korean Chemical Society*, 29(3), pp. 590–594. doi: 10.5012/bkcs.2008.29.3.590.

Kim, J. and Amar, S. (2006) 'Periodontal disease and systemic conditions: a bidirectional relationship.', *Odontology*, 94(1), pp. 10–21. doi: 10.1007/s10266-006-0060-6.

Kirisits, M. J. *et al.* (2007) 'Influence of the hydrodynamic environment on quorum sensing in Pseudomonas aeruginosa biofilms', *Journal of Bacteriology*, 189(22), pp. 8357–8360. doi: 10.1128/JB.01040-07.

Klemm, P. and Schembri, M. A. (2000) 'Bacterial adhesins: Function and structure', *International Journal of Medical Microbiology*, 290(1), pp. 27–35. doi: 10.1016/S1438-4221(00)80102-2.

Klug, B. *et al.* (2016) 'From Mouth to Model: Combining in vivo and in vitro Oral Biofilm Growth', *Frontiers in Microbiology*, 7, p. 1448. doi: 10.3389/fmicb.2016.01448.

Kohyama, K. *et al.* (2004) 'Effects of sample hardness on human chewing force: A model study using silicone rubber', *Archives of Oral Biology*, 49(10), pp. 805–816. doi: 10.1016/j.archoralbio.2004.04.006.

Kolenbrander, P. E. *et al.* (2010) 'Oral multispecies biofilm development and the key role of cell–cell distance', *Nature Reviews Microbiology*, 8(7), pp. 471–480. doi: 10.1038/nrmicro2381.

Kolenbrander, P. E., Andersen, R. N. and Moore, L. V (1989) 'Coaggregation of Fusobacterium nucleatum, Selenomonas flueggei, Selenomonas infelix, Selenomonas noxia, and Selenomonas sputigena with strains from 11 genera of oral bacteria.', *Infection and immunity*, 57(10), pp. 3194–203. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2777378 (Accessed: 2 May 2019).

Koo, H. *et al.* (2003) 'Inhibition of Streptococcus mutans biofilm accumulation and polysaccharide production by apigenin and tt-farnesol', *Journal of Antimicrobial Chemotherapy*, 52(5), pp. 782–789. doi: 10.1093/jac/dkg449.

Koopman, J. E. *et al.* (2014) 'Stability and Resilience of Oral Microcosms Toward Acidification and Candida Outgrowth by Arginine Supplementation', *Microbial Ecology*, 69(2), pp. 422–433. doi: 10.1007/s00248-014-0535-x.

Kumar, S. B. (2017) 'Chlorhexidine Mouthwash- A Review', 9(9), pp. 1450–1452.

De La Fuente, L. *et al.* (2007) 'Assessing adhesion forces of type I and type IV pili of Xylella fastidiosa bacteria by use of a microfluidic flow chamber', *Applied and Environmental Microbiology*, 73(8), pp. 2690–2696. doi: 10.1128/AEM.02649-06.

Lappin-Scott, H. M. and Bass, C. (2001) 'Biofilm formation: Attachment, growth, and detachment of microbes from surfaces', *American Journal of Infection Control*, 29(4), pp. 250–251. doi: 10.1067/mic.2001.115674.

Latimer, J. *et al.* (2015) 'Antibacterial and anti-biofilm activity of mouthrinses containing cetylpyridinium chloride and sodium fluoride.', *BMC microbiology*, 15, p. 169. doi: 10.1186/s12866-015-0501-x.

Laursen, M. F., Dalgaard, M. D. and Bahl, M. I. (2017) 'Genomic GC-content affects the accuracy of 16S rRNA gene sequencing bsed microbial profiling due to PCR bias', *Frontiers in Microbiology*, 8(OCT), pp. 1–8. doi: 10.3389/fmicb.2017.01934.

Lazarevic, V. *et al.* (2009) 'Metagenomic study of the oral microbiota by Illumina high-throughput sequencing', *Journal of Microbiological Methods*, 79(3), pp. 266–271. doi: 10.1016/j.mimet.2009.09.012.

Lee, H. J. *et al.* (2019) 'Tooth Loss Predicts Myocardial Infarction, Heart Failure, Stroke, and Death', *Journal of Dental Research*, 98(2), pp. 164–170. doi: 10.1177/0022034518814829.

Lee, Y.-E. *et al.* (2008) 'Morphological Changes in Streptococcus mutans After Chewing Gum Containing Xylitol for Twelve Months', *Current Microbiology*, 58(4), pp. 332–337. doi: 10.1007/s00284-008-9332-4.

Leme, A. F. P. *et al.* (2006) 'The Role of Sucrose in Cariogenic Dental Biofilm Formation—New Insight', *J Dent Res*, 85(10), pp. 878–887.

Leszczyńska, K. *et al.* (2013) 'Antibacterial activity of the human host defence peptide LL-37 and selected synthetic cationic lipids against bacteria associated with oral and upper respiratory tract infections', *Journal of Antimicrobial Chemotherapy*, 68(3), pp. 610–618. doi: 10.1093/jac/dks434.

Li, X. *et al.* (2000) *Systemic Diseases Caused by Oral Infection*. Available at: http://cmr.asm.org/ (Accessed: 30 May 2019).

Li, X. *et al.* (2021) 'Saliva-derived microcosm biofilms grown on different oral surfaces in vitro', *npj Biofilms and Microbiomes*, 7(1). doi: 10.1038/s41522-021-00246-z.

Li, Y. H. *et al.* (2001) 'Natural genetic transformation of streptococcus mutans growing in biofilms',

Journal of Bacteriology, 183(3), pp. 897–908. doi: 10.1128/JB.183.3.897-908.2001.

Löe, H. (1969) 'Present Day Status and Direction for Future Research on the Etiology and Prevention of Periodontal Disease', *Journal of Periodontology*, 40(12), pp. 678–682. doi: 10.1902/jop.1969.40.12.678.

Löe, H. and Rindom Schiøtt, C. (1970) 'The effect of mouthrinses and topical application of chlorhexidine on the development of dental plaque and gingivitis in man', *Journal of Periodontal Research*, pp. 79–83. doi: 10.1111/j.1600-0765.1970.tb00696.x.

Ma, L. *et al.* (2009) 'Assembly and development of the Pseudomonas aeruginosa biofilm matrix', *PLoS Pathogens*, 5(3). doi: 10.1371/journal.ppat.1000354.

Mao, X. *et al.* (2020) 'Cetylpyridinium chloride: Mechanism of action, antimicrobial efficacy in biofilms, and potential risks of resistance', *Antimicrobial Agents and Chemotherapy*, 64(8), pp. 1–14. doi: 10.1128/AAC.00576-20.

Del Mar Lleo, M. *et al.* (2000) 'mRNA detection by reverse transcription-PCR for monitoring viability over time in an Enterococcus faecalis viable but nonculturable population maintained in a laboratory microcosm', *Applied and Environmental Microbiology*, 66(10), pp. 4564–4567. doi: 10.1128/AEM.66.10.4564-4567.2000.

Marsh, P. (2006) 'Dental plaque as a biofilm and a microbial community - implications for health and disease.', *BMC oral health*, 6 Suppl 1(Suppl 1), p. S14. doi: 10.1186/1472-6831-6-S1-S14.

Marsh, P. D. (1994) 'Microbial ecology of dental plaque and its significance in health and disease.', *Advances in dental research*, 8(2), pp. 263–271. doi: 10.1177/08959374940080022001.

Martínez-Pabón, M. C. and Ortega-Cuadros, M. (2020) 'Thymol, menthol and eucalyptol as agents for microbiological control in the oral cavity: A scoping review', *Revista Colombiana de Ciencias Químico-Farmacéuticas*, 49(1), pp. 44–69. doi: 10.15446/rcciquifa.v49n1.87006.

Mascarenhas, P., Fatela, B. and Barahona, I. (2014) 'Effect of diabetes mellitus type 2 on salivary glucose - A systematic review and meta-analysis of observational studies', *PLoS ONE*, 9(7). doi: 10.1371/journal.pone.0101706.

Mat Ludin, C. M. and Md Radzi, J. (2001) 'The antimicrobial activity of different mouthwashes in malaysia.', *The Malaysian journal of medical sciences : MJMS*, 8(2), pp. 14–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22893755 (Accessed: 29 April 2019).

Mathews, J. P. (2009) *Chicle: The Chewing Gum of the Americas, from the Ancient Maya to*

Bibliography

William Wrigley.

Mathur, S. *et al.* (2011) 'Chlorhexidine: The gold standard in chemical plaque control', *National Journal of Physiology, Pharmacy and Pharmacology*, 1(2), pp. 45–50.

McCormack, M. G. *et al.* (2015) 'Staphylococcus aureus and the oral cavity: An overlooked source of carriage and infection?', *American Journal of Infection Control*, 43(1), pp. 35–37. doi: 10.1016/j.ajic.2014.09.015.

Merchant, A. T. *et al.* (2018) 'Periodontal treatment among mothers with mild to moderate periodontal disease and preterm birth: Reanalysis of OPT trial data accounting for selective survival', *International Journal of Epidemiology*, 47(5), pp. 1670–1678. doi: 10.1093/ije/dyy089.

Millsop, J. W. and Fazel, N. (2016) 'Oral candidiasis', *Clinics in Dermatology*, 34(4), pp. 487–494. doi: 10.1016/j.clindermatol.2016.02.022.

Mirzoeva, O. K., Grishanin, R. N. and Calder, P. C. (1997) 'Antimicrobial action of propolis and some of its components: The effects on growth, membrane potential and motility of bacteria', *Microbiological Research*, 152(3), pp. 239–246. doi: 10.1016/S0944-5013(97)80034-1.

Monti M., Berti E, Carminati G, and C. m (1983) 'Occupational and cosmetic dermatitis from propolis', p. 163. doi: 10.1111/j.1600-0536.1983.tb04341.x.

Moore, L. V. H. *et al.* (1987) 'Bacteriology of Human Gingivitis', *Journal of Dental Research*, 66(5), pp. 989–995. doi: 10.1177/00220345870660052401.

Murugkar, P. *et al.* (2023) 'Identification of a growth factor required for culturing specific fastidious oral bacteria', *Journal of Oral Microbiology*, 15(1). doi: 10.1080/20002297.2022.2143651.

Nath, S. G. and Raveendran, R. (2013) 'Microbial dysbiosis in periodontitis.', *Journal of Indian Society of Periodontology*, 17(4), pp. 543–5. doi: 10.4103/0972-124X.118334.

Nayak, P. A., Nayak, U. A. and Khandelwal, V. (2014) 'The effect of xylitol on dental caries and oral flora', *Clinical, Cosmetic and Investigational Dentistry*, 6, p. 89. doi: 10.2147/CCIDE.S55761.

Nazzaro, F. *et al.* (2013) 'Effect of essential oils on pathogenic bacteria', *Pharmaceuticals*, 6(12), pp. 1451–1474. doi: 10.3390/ph6121451.

Ng, W. and Tonzetich, J. (1984) 'Effect of Hydrogen Sulfide and Methyl Mercaptan on the Permeability of Oral Mucosa', *Journal of Dental Research*, 63(7), pp. 994–997. doi:

10.1177/00220345840630071701.

NHS (2018) *How to keep your teeth clean*. Available at: https://www.nhs.uk/live-well/healthy-body/how-to-keep-your-teeth-clean/ (Accessed: 28 May 2019).

Noel, D. J., Keevil, C. W. and Wilks, S. A. (2021) 'Synergism versus Additivity: Defining the Interactions between Common Disinfectants', *mBio*, 12(5). doi: 10.1128/mBio.02281-21.

O'Toole, G., Kaplan, H. B. and Kolter, R. (2000) 'Biofilm Formation as Microbial Development', *Annual Review of Microbiology*, 54(1), pp. 49–79. doi: 10.1146/annurev.micro.54.1.49.

Odds, F. C. (1987) 'Candida infections: An overview', *Critical Reviews in Microbiology*, 15(1), pp. 1–5. doi: 10.3109/10408418709104444.

Offenbacher, S. *et al.* (2009) 'Effects of periodontal therapy on rate of preterm delivery: A randomized controlled trial', *Obstetrics and Gynecology*, 114(3), pp. 551–559. doi: 10.1097/AOG.0b013e3181b1341f.

Oliver, J. (2000) 'The viable but nonculturable state and cellular resuscitation', *Microbial biosystems: new frontiers. Atlantic Canada …*, pp. 723–730. Available at: http://plato.acadiau.ca/isme/Symposium23/oliver.PDF.

Ollie Yiru Yu, Irene Shuping Zhao, May Lei Mei, E. C.-M. L. and C.-H. C. (2017) 'Dental Biofilm and Laboratory Microbial Culture Models for Cariology Research', *Dentistry Journal*, 5(2), p. 21. doi: 10.3390/dj5020021.

Ouhayoun, J. P. (2003) 'Penetrating the plaque biofilm: Impact of essential oil mouthwash', *Journal of Clinical Periodontology*, 30(SUPPL. 5), pp. 10–12. doi: 10.1034/j.1600-051X.30.s5.4.x.

Page, R. C. (1986) 'Gingivitis*', *Journal of Clinical Periodontology*, 13(5), pp. 345–355. doi: 10.1111/j.1600-051X.1986.tb01471.x.

Pancu, G. *et al.* (2017) *THE EFFECT OF XYLITOL-BASED ORAL HYGIENE PRODUCTS ON SALIVA PARAMETERS AND BACTERIAL BIOFILM CARIOACTIVITY*, *Romanian Journal of Oral Rehabilitation*. Available at: http://www.rjor.ro/wp-content/uploads/2017/07/THE-EFFECT-OF-XYLITOL-BASED-ORAL-HYGIENE-PRODUCTS-ON-SALIVA-PARAMETERS-AND-BACTERIAL-BIOFILM-CARIOACTIVITY-rjor-nr-2.2017.pdf (Accessed: 30 April 2019).

Papagianni, M. (2007) 'Advances in citric acid fermentation by Aspergillus niger: Biochemical aspects, membrane transport and modeling', *Biotechnology Advances*, 25(3), pp. 244–263. doi: 10.1016/j.biotechadv.2007.01.002.

Park, H.-R. and Park, C.-H. (2017) 'Spectroscopic Study on the Anti-oxidant Properties of Flavonoid Galangin', *Asian Journal of Beauty and Cosmetology*, 15(4), pp. 411–419. doi: 10.20402/ajbc.2017.0146.

Pasquaroli, S. *et al.* (2013) 'Antibiotic pressure can induce the viable but non-culturable state in Staphylococcus aureus growing in biofilms', *Journal of Antimicrobial Chemotherapy*, 68(8), pp. 1812–1817. doi: 10.1093/jac/dkt086.

Paulussen, C. *et al.* (2017) 'Ecology of aspergillosis: insights into the pathogenic potency of Aspergillus fumigatus and some other Aspergillus species', *Microbial Biotechnology*, 10(2), pp. 296–322. doi: 10.1111/1751-7915.12367.

Peres, M. A. *et al.* (2019) 'Oral diseases: a global public health challenge', *The Lancet*, 394(10194), pp. 249–260. doi: 10.1016/S0140-6736(19)31146-8.

Plaper, A. *et al.* (2003) 'Characterization of quercetin binding site on DNA gyrase', *Biochemical and Biophysical Research Communications*, 306(2), pp. 530–536. doi: 10.1016/S0006-291X(03)01006-4.

Pootong, A., Norrapong, B. and Cowawintaweewat, S. (2017) 'Antifungal activity of cinnamaldehyde against Candida albicans', *Southeast Asian Journal of Tropical Medicine and Public Health*, 48(1), pp. 150–158.

Pratten, J. (2007) 'Growing Oral Biofilms in a Constant Depth Film Fermentor (CDFF)', *Current Protocols in Microbiology*, 6(1), pp. 1–18. doi: 10.1002/9780471729259.mc01b05s6.

Progulske-Fox, A. *et al.* (2022) 'VBNC, previously unrecognized in the life cycle of Porphyromonas gingivalis?', *Journal of Oral Microbiology*, 14(1). doi: 10.1080/20002297.2021.1952838.

Przybyłek, I. and Karpiński, T. M. (2019) 'Antibacterial properties of propolis', *Molecules*, 24(11), pp. 11–13. doi: 10.3390/molecules24112047.

PubChem (2023) *Chlorhexidine Gluconate compound summary*. Available at: https://pubchem.ncbi.nlm.nih.gov/compound/Chlorhexidine-gluconate.

Puth, S. *et al.* (2019) 'A built-in adjuvant-engineered mucosal vaccine against dysbiotic periodontal diseases', *Mucosal Immunology*, 12(2), pp. 565–579. doi: 10.1038/s41385-018-0104-6.

Quintas, V. *et al.* (2014) 'In situ antimicrobial activity on oral biofilm: essential oils vs. 0.2 % chlorhexidine', *Clinical Oral Investigations*, 19(1), pp. 97–107. doi: 10.1007/s00784-014-1224-3.

Quirynen, M. *et al.* (2001) 'Bacterial survival rate on tooth-and interdental brushes in relation to the use of toothpaste', *Journal of Clinical Periodontology*, 28(12), pp. 1106–1114. doi: 10.1034/j.1600-051X.2001.281204.x.

Quirynen, M. *et al.* (2003) 'Can Toothpaste or a Toothbrush with Antibacterial Tufts Prevent Toothbrush Contamination?', *Journal of Periodontology*, 74(3), pp. 312–322. doi: 10.1902/jop.2003.74.3.312.

Quirynen, M. *et al.* (2008) 'Bacterial survival rate on tooth- and interdental brushes in relation to the use of toothpaste', *Journal of Clinical Periodontology*, 28(12), pp. 1106–1114. doi: 10.1111/j.1600-051X.2001.281204.x.

Rafeek, R. *et al.* (2019) 'Xylitol and sorbitol effects on the microbiome of saliva and plaque', *Journal of Oral Microbiology*, 11(1), p. 1536181. doi: 10.1080/20002297.2018.1536181.

Ramamurthy, T. *et al.* (2014) 'Current perspectives on viable but non-culturable (VBNC) pathogenic bacteria', *Frontiers in Public Health*, 2(JUL), pp. 1–9. doi: 10.3389/fpubh.2014.00103.

Ratcliff, P. A. and Johnson, P. W. (1999) 'The Relationship Between Oral Malodor, Gingivitis, and Periodontitis. A Review', *Journal of Periodontology*, 70(5), pp. 485–489. doi: 10.1902/jop.1999.70.5.485.

Ravaioli, S. *et al.* (2020) 'Various biofilm matrices of the emerging pathogen Staphylococcus lugdunensis: exopolysaccharides, proteins, eDNA and their correlation with biofilm mass', *Biofouling*, 36(1), pp. 86–100. doi: 10.1080/08927014.2020.1716217.

Reactor, B. (2017) 'BioSurface Technologies Corporation Operator s Manual CDC bioreactor', (Ibr 500).

Rezaei, T. *et al.* (2023) 'Factors Associated with Streptococcus mutans Pathogenicity in the Oral Cavity', *Biointerface Research in Applied Chemistry*, 13(4), pp. 1–19. doi: 10.33263/BRIAC134.368.

RindomSchiostt, C. *et al.* (1976) 'Two years oral use of chlorhexidine in man.', *Journal of Periodontal Research*, 11(3), pp. 153–157. doi: 10.1111/j.1600-0765.1976.tb00063.x.

Romero, M. *et al.* (2019) 'Propolis polyphenolic compounds affect the viability and structure of Helicobacter pylori in vitro', *Revista Brasileira de Farmacognosia*, 29(3), pp. 325–332. doi: 10.1016/j.bjp.2019.03.002.

Roszak, D. B., Grimes, D. J. and Colwell, R. R. (1984) 'Viable but nonrecoverable stage of Salmonella enteritidis in aquatic systems', *Canadian Journal of Microbiology*, 30(3), pp. 334–338.

doi: 10.1139/m84-049.

Rudney, J. D. *et al.* (2012) 'A Reproducible Oral Microcosm Biofilm Model for Testing Dental Materials'. doi: 10.1111/j.1365-2672.2012.05439.x.

Rusconi, R. *et al.* (2010) 'Laminar flow around corners triggers the formation of biofilm streamers', *Journal of the Royal Society Interface*, 7(50), pp. 1293–1299. doi: 10.1098/rsif.2010.0096.

Russell, M. A. H., Raw, M. and Jarvis, M. J. (1980) 'Clinical use of nicotine chewing-gum', *British Medical Journal*, 280(6231), pp. 1599–1602. doi: 10.1136/bmj.280.6231.1599.

S K Filoche, K J Soma, C. H. S. (2007) 'Caries-related plaque microcosm biofilms developed in microplates', *Wiley Online Library*.

Saini, R., Saini, S. and Saini, S. R. (2010) 'Periodontitis: A risk for delivery of premature labor and low-birth-weight infants.', *Journal of natural science, biology, and medicine*, 1(1), pp. 40–2. doi: 10.4103/0976-9668.71672.

Sajjan P, Laxminarayan N, Kar PP, S. M. (2016) 'Chlorhexidine as an Antimicrobial Agent in Dentistry – A Review', *Oral Health and Dental Management*, 15(2), pp. 93–100.

Sauer, F. G. *et al.* (2000) 'Bacterial pili: Molecular mechanisms of pathogenesis', *Current Opinion in Microbiology*, 3(1), pp. 65–72. doi: 10.1016/S1369-5274(99)00053-3.

Sauer, K. *et al.* (2022) 'The biofilm life cycle: expanding the conceptual model of biofilm formation', *Nature Reviews Microbiology*, 20(10), pp. 608–620. doi: 10.1038/s41579-022-00767-0.

Saunders, K. A. and Greenman, J. (2000) 'The formation of mixed culture biofilms of oral species along a gradient of shear stress', *Journal of Applied Microbiology*, 89(4), pp. 564–572. doi: 10.1046/j.1365-2672.2000.01148.x.

Scannapieco, F. A., Solomon, L. and Wadenya, R. O. (1994) 'Emergence in Human Dental Plaque and Host Distribution of Amylase-binding Streptococci', *Journal of Dental Research*, 73(10), pp. 1627–1635. doi: 10.1177/00220345940730100701.

Shanahan, F., Ghosh, T. S. and O'Toole, P. W. (2021) 'The Healthy Microbiome—What Is the Definition of a Healthy Gut Microbiome?', *Gastroenterology*, 160(2), pp. 483–494. doi: 10.1053/j.gastro.2020.09.057.

Shibuya, K. *et al.* (2004) 'Pathophysiology of pulmonary aspergillosis', *Journal of Infection and Chemotherapy*, 10(3), pp. 138–145. doi: 10.1007/s10156-004-0315-5.

Simons, D. *et al.* (2001) 'The effect of chlorhexidine acetate/xylitol chewing gum on the plaque and gingival indices of elderly occupants in residential homes: A 1-year clinical trial', *Journal of Clinical Periodontology*, 28(11), pp. 1010–1015. doi: 10.1034/j.1600-051x.2001.281104.x.

Singhal, R. *et al.* (2020) 'Antimicrobial and antibiofilm effect of cranberry extract on Streptococcus mutans and Lactobacillus acidophilus: An in vitro study', *International Journal of Clinical Pediatric Dentistry*, 13(1), pp. 11–15. doi: 10.5005/jp-journals-10005-1707.

Siqueira, W. L., Custodio, W. and McDonald, E. E. (2012) 'New insights into the composition and functions of the acquired enamel pellicle', *Journal of Dental Research*, 91(12), pp. 1110–1118. doi: 10.1177/0022034512462578.

Socransky, S. S. *et al.* (1998) 'Microbial complexes in subgingival plaque', *Journal of Clinical Periodontology*, 25(2), pp. 134–144. doi: 10.1111/j.1600-051X.1998.tb02419.x.

Söderling, E. *et al.* (2011) 'The Effect of xylitol on the composition of the oral flora: A pilot study', *European Journal of Dentistry*, 5(1), pp. 24–31. doi: 10.1055/s-0039-1698855.

Sokolonski, A. R. *et al.* (2021) 'Activity of antifungal drugs and Brazilian red and green propolis extracted with different methodologies against oral isolates of Candida spp.', *BMC Complementary Medicine and Therapies*, 21(1), pp. 1–14. doi: 10.1186/s12906-021-03445-5.

Sousa, V. *et al.* (2022) 'Oral Microcosm Biofilms Grown under Conditions Progressing from Peri-Implant Health, Peri-Implant Mucositis, and Peri-Implantitis', *International Journal of Environmental Research and Public Health*, 19(21). doi: 10.3390/ijerph192114088.

Speciale, A. *et al.* (2006) 'Antibacterial activity of Propolis and its active principles alone and in combination with macrolides, beta-lactams and fluoroquinolones against microorganisms responsible for respiratory infections', *Journal of Chemotherapy*, 18(2), pp. 164–171. doi: 10.1179/joc.2006.18.2.164.

Sreenivasan, P. K., Haraszthy, V. I. and Zambon, J. J. (2013) 'Antimicrobial efficacy of 0·05% cetylpyridinium chloride mouthrinses', *Letters in Applied Microbiology*, 56(1), pp. 14–20. doi: 10.1111/lam.12008.

Stefanakis, A. *et al.* (2014) 'Treatment Processes in VFCWs', *Vertical Flow Constructed Wetlands*, pp. 57–84. doi: 10.1016/B978-0-12-404612-2.00005-2.

Steiner, M. *et al.* (2009) 'In vitro evaluation of a mechanical testing chewing simulator', *Dental Materials*, 25(4), pp. 494–499. doi: 10.1016/j.dental.2008.09.010.

Stewart, P. S. (2014) 'Biophysics of biofilm infection', *Pathogens and Disease*, 70(3), pp. 212–218. doi: 10.1111/2049-632X.12118.

Stoodley, P., Debeer, D. and Lewandowski, Z. (1994) *Liquid Flow in Biofilm Systems*, *APPLIED AND ENVIRONMENTAL MICROBIOLOGY*. Available at: http://aem.asm.org/ (Accessed: 30 May 2019).

Sudhakara, P. *et al.* (2018) 'Oral dysbiotic communities and their implications in systemic diseases', *Dentistry Journal*, 6(2), pp. 1–14. doi: 10.3390/dj6020010.

Suppiger, S. *et al.* (2020) 'Tolerance and persister formation in oral streptococci', *Antibiotics*, 9(4), pp. 1–9. doi: 10.3390/antibiotics9040167.

Takahashi, K. *et al.* (2003) 'A Pilot Study on Antiplaque Effects of Mastic Chewing Gum in the Oral Cavity', *Journal of Periodontology*, 74(4), pp. 501–505. doi: 10.1902/jop.2003.74.4.501.

Tavernier, S. *et al.* (2017) 'Community composition determines activity of antibiotics against multispecies biofilms', *Antimicrobial Agents and Chemotherapy*, 61(9), pp. 1–12. doi: 10.1128/AAC.00302-17.

Telford, J. L. *et al.* (2006) 'Pili in Gram-positive pathogens', *Nature Reviews Microbiology*, 4(7), pp. 509–519. doi: 10.1038/nrmicro1443.

Tellefsen, G. *et al.* (1996) 'Use of Chlorhexidine Chewing Gum Significantly Reduces Dental Plaque Formation Compared to Use of Similar Xylitol and Sorbitol Products', *Journal of Periodontology*, 67(3), pp. 181–183. doi: 10.1902/jop.1996.67.3.181.

Thurnheer, T. and Belibasakis, G. N. (2018) 'Effect of sodium fluoride on oral biofilm microbiota and enamel demineralization', *Archives of Oral Biology*, 89, pp. 77–83. doi: 10.1016/j.archoralbio.2018.02.010.

Thurnheer, T., Belibasakis, G. N. and Bostanci, N. (2014) 'Colonisation of gingival epithelia by subgingival biofilms in vitro: Role of "red complex" bacteria', *Archives of Oral Biology*, 59(9), pp. 977–986. doi: 10.1016/j.archoralbio.2014.05.023.

Tolker-Nielsen, T. (2015) 'Biofilm Development', *Microbiology Spectrum*, 3(2), p. 100043. doi: 10.1128/microbiolspec.

Tseng, B. S. *et al.* (2013) 'The extracellular matrix protects Pseudomonas aeruginosa biofilms by limiting the penetration of tobramycin', *Environmental Microbiology*, 23(1), pp. 1–7. doi: 10.1111/1462-2920.12155.The.

U. Lendenmann' *, J. Grogan1, F. G. O. (2000) 'Saliva and Dental Pellicle — A Review', *Advances in Dental Research*.

US EPA, O. (2017) 'Methods and Guidance for Testing the Efficacy of Antimicrobial Products Against Biofilms on Hard, Non-Porous Surfaces'. Available at: https://www.epa.gov/pesticide-analytical-methods/methods-and-guidance-testing-efficacy-antimicrobial-products-against (Accessed: 3 June 2019).

Vartoukian, S. R. *et al.* (2016) 'In Vitro Cultivation of "Unculturable" Oral Bacteria, Facilitated by Community Culture and Media Supplementation with Siderophores', *PLOS ONE*. Edited by Z. Wen, 11(1), p. e0146926. doi: 10.1371/journal.pone.0146926.

Vats, N. and Lee, S. F. (2000) 'Active detachment of Streptococcus mutans cells adhered to epon-hydroxylapatite surfaces coated with salivary proteins in vitro', *Archives of Oral Biology*, 45(4), pp. 305–314. doi: 10.1016/S0003-9969(99)00139-9.

Veloz, J. J., Alvear, M. and Salazar, L. A. (2019) 'Antimicrobial and Antibiofilm Activity against Streptococcus mutans of Individual and Mixtures of the Main Polyphenolic Compounds Found in Chilean Propolis', *BioMed Research International*, 2019. doi: 10.1155/2019/7602343.

Verma, D., Garg, P. K. and Dubey, A. K. (2018) 'Insights into the human oral microbiome', *Archives of Microbiology*, 200(4), pp. 525–540. doi: 10.1007/s00203-018-1505-3.

Vlachojannis, C. *et al.* (2015) 'A preliminary investigation on the antimicrobial activity of Listerine®, its components, and of mixtures thereof', *Phytotherapy Research*, 29(10), pp. 1590–1594. doi: 10.1002/ptr.5399.

Wang, L. *et al.* (2019) 'The complex essential oils highly control the toxigenic fungal microbiome and major mycotoxins during storage of maize', *Frontiers in Microbiology*, 10(JULY), pp. 1–13. doi: 10.3389/fmicb.2019.01643.

Watson, S. P., Clements, M. O. and Foster, S. J. (1998) 'Characterization of the starvation-survival response of Staphylococcus aureus', *Journal of Bacteriology*, 180(7), pp. 1750–1758. doi: 10.1128/jb.180.7.1750-1758.1998.

Weichart, D. and Kjelleberg, S. (1996) 'Stress resistance and recovery potential of culturable and viable but nonculturable cells of Vibrio vulnificus', *Microbiology*, 142(4), pp. 845–853. doi: 10.1099/00221287-142-4-845.

Wessel, S. W. *et al.* (2015) 'Quantification and Qualification of Bacteria Trapped in Chewed Gum',

PLOS ONE. Edited by A. Al-Ahmad, 10(1), p. e0117191. doi: 10.1371/journal.pone.0117191.

West, N. X. *et al.* (2017) 'Erosion protection benefits of stabilized SnF2 dentifrice versus an arginine–sodium monofluorophosphate dentifrice: results from in vitro and in situ clinical studies', *Clinical Oral Investigations*, 21(2), pp. 533–540. doi: 10.1007/s00784-016-1905-1.

Wilking, J. N. *et al.* (2013) 'Liquid transport facilitated by channels in Bacillus subtilis biofilms', *Proceedings of the National Academy of Sciences of the United States of America*, 110(3), pp. 848–852. doi: 10.1073/pnas.1216376110.

Wingender, J., Neu, T. R. and Flemming, H.-C. (1999) 'What are Bacterial Extracellular Polymeric Substances?', *Microbial Extracellular Polymeric Substances*, pp. 1–19. doi: 10.1007/978-3-642-60147-7_1.

Von Wintersdorff, C. J. H. *et al.* (2016) 'Dissemination of antimicrobial resistance in microbial ecosystems through horizontal gene transfer', *Frontiers in Microbiology*, 7(FEB), pp. 1–10. doi: 10.3389/fmicb.2016.00173.

Wojtyczka, R. D. *et al.* (2013) 'Susceptibility of Staphylococcus aureus clinical isolates to propolis extract alone or in combination with antimicrobial drugs', *Molecules*, 18(8), pp. 9623–9640. doi: 10.3390/molecules18089623.

Wood, T. K., Knabel, S. J. and Kwan, B. W. (2013) 'Bacterial persister cell formation and dormancy.', *Applied and environmental microbiology*, 79(23), pp. 7116–21. doi: 10.1128/AEM.02636-13.

Woodford, D. W. and Lesko, L. J. (1981) 'Relative bioavailability of aspirin gum', *Journal of Pharmaceutical Sciences*, 70(12), pp. 1341–1343. doi: 10.1002/jps.2600701213.

Worthington, H. V. *et al.* (2019) 'Home use of interdental cleaning devices, in addition to toothbrushing, for preventing and controlling periodontal diseases and dental caries', *Cochrane Database of Systematic Reviews*. John Wiley and Sons Ltd. doi: 10.1002/14651858.CD012018.pub2.

Wright, M. H., Adelskov, J. and Greene, A. C. (2017) 'Bacterial DNA Extraction Using Individual Enzymes and Phenol/Chloroform seperation', *Journal of Microbiology & Biology Education*, 18(2), pp. 1–3.

Wu, H. and Fives-Taylor, P. M. (2001) 'Molecular strategies for fimbrial expression and assembly', *Critical Reviews in Oral Biology and Medicine*, 12(2), pp. 101–115. doi:

10.1177/10454411010120020101.

Wu, Y. F. *et al.* (2022) 'Xylitol-Containing Chewing Gum Reduces Cariogenic and Periodontopathic Bacteria in Dental Plaque—Microbiome Investigation', *Frontiers in Nutrition*, 9(May), pp. 1–12. doi: 10.3389/fnut.2022.882636.

Xu, X., Ou, Z. M. and Wu, C. D. (2018) 'Growth Media Affect Assessment of Antimicrobial Activity of Plant-Derived Polyphenols', *BioMed Research International*, 2018. doi: 10.1155/2018/8308640.

Yang, X. *et al.* (2018) 'Pinocembrin–lecithin complex: Characterization, solubilization, and antioxidant activities', *Biomolecules*, 8(2). doi: 10.3390/biom8020041.

Ye, C. *et al.* (2020) 'Characterization and potential mechanisms of highly antibiotic tolerant VBNC Escherichia coli induced by low level chlorination', *Scientific Reports*, 10(1), pp. 1–11. doi: 10.1038/s41598-020-58106-3.

Yitzhaki, S. *et al.* (2018) 'Microbiome associated with denture malodour', *Journal of Breath Research*, 12(2), pp. 0–20. doi: 10.1088/1752-7163/aa95e0.

Yoo, S., Murata, R. M. and Duarte, S. (2011) 'Antimicrobial traits of tea- and cranberry-derived polyphenols against streptococcus mutans', *Caries Research*, 45(4), pp. 327–335. doi: 10.1159/000329181.

Young, N. W. G. and O'Sullivan, G. R. (2011) *The influence of ingredients on product stability and shelf life*, *Food and Beverage Stability and Shelf Life*. Woodhead Publishing Limited. doi: 10.1533/9780857092540.1.132.

Yung-Hua, D. G. C. et. al. (2002) 'A Quorum-Sensing Signaling System Essential for Genetic Competence in Streptococcus mutans Is Involved in Biofilm Formation', *Journal for bacteriology*, 184(10), pp. 2699–2708. doi: 10.1128/JB.184.10.2699.

Zaura, E. *et al.* (2009) 'Defining the healthy "core microbiome" of oral microbial communities', *BMC Microbiology*, 9(1), p. 259. doi: 10.1186/1471-2180-9-259.

Zeng, P. *et al.* (2009) 'Solubility properties of chlorhexidine salts', *Drug Development and Industrial Pharmacy*, 35(2), pp. 172–176. doi: 10.1080/03639040802220318.

Zhang, K. *et al.* (2009) 'Effects of quorum sensing on cell viability in Streptococcus mutans biofilm formation', *Biochemical and Biophysical Research Communications*, 379(4), pp. 933–938. doi: 10.1016/j.bbrc.2008.12.175.

Zheng, Sherry *et al.* (2021) 'Implication of Surface Properties, Bacterial Motility, and Hydrodynamic Conditions on Bacterial Surface Sensing and Their Initial Adhesion', *Frontiers in Bioengineering and Biotechnology*, 9(February), pp. 1–22. doi: 10.3389/fbioe.2021.643722.

Zheng, Shaowen *et al.* (2021) 'Porphyromonas gingivalis survival skills: Immune evasion', *Journal of Periodontal Research*, 56(6), pp. 1007–1018. doi: 10.1111/jre.12915.