CRANFIELD UNIVERSITY

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Screening of Novel Compounds for Inhibiting Bacteria Involved in Dental Caries

Cranfield Health

MSc by Research
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ABSTRACT

Dental caries is the most common infectious disease affecting humans. The main causative agents of this disease are bacteria especially group of streptococcal species. This study has examined the potential of using essential oils/antioxidants to control the growth of *Streptococcus mutans* and *Streptococcus oralis* in vitro in tryptone soya broth and a saliva medium in the presence and absence of sodium fluoride. A total of 9 essential oils and three antioxidants were screened using a clearing zone method. This showed that two essential oils (clove leaf, and cinnamon oils) and two antioxidants (butyl hydroxy anisole and propyl gallate) were the most effective. These were then tested at up to 10% concentration for inhibition of cell viability of both *S.mutans* and *S.oralis.* The ED_{50} and ED_{90} concentrations of these four compounds were determined. Studies were then carried out to examine the potential of integrating these compounds with sodium fluoride for inhibition of these two oral bacteria. Concentrations of 0.5 and 1% of the essential oils/antioxidants + 2000ppm sodium fluoride was able to completely inhibit growth of populations of both bacteria in TSB medium. Studies in an artificial saliva medium showed that these mixture were still effective resulting in >75% decrease in populations after treatment for 24 hrs. Ecological studies using the Bioscreen system showed that *S.mutans* and *S.oralis* could grow well at pH 5-7, but much more slowly at pH 4 as indicated by the times to detection and the increase in lag times prior to growth initiation. An increase in NaCl concentration from 1 to 6% (=0.999 to 0.97 water activity) showed a similar result for both bacteria.

Keywords: Essential oils, antioxidants, *Streptococcus mutans*, *S.oralis*, dental caries, control of growth, sodium fluoride, ecology, pH, water activity

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

TSB	Tryptone Soya Broth
TSA	Tryptone Soya Agar
CFUs	Colony forming units
S	Streptococcus
ECC	Early Childhood Caries
OD	optical density
TTD	Time To Detection
GTF	glycosyltransferases
BHT	Butylated hydroxy toluene
BHA	Butylated hydroxy anisole
PG	Propyl gallate
ppm	Parts per million
ul	microliter
NaF	Sodium fluoride
NaCl	Sodium chloride
KCl	Potassium chloride
$CaCl_2.2H_2O$	Calcium chloride dihydrate
$NaH_2PO_4\ 2H_2O$	Sodium phosphate dihydrate
NH_2CONH_2	Urea
a_w	Water activity
ED_{50}	Effective Dose , 50 concentration need to give inhibit availability by 50%
ED_{90}	Effective Dose , 90 concentration need to give inhibit availability by 90%

Chapter one

1 General introduction and Literature Review

1.1 General Introduction

Tooth decay is caused by poor nutrition associated with a deficiency of vitamins, minerals, and other nutrients that the body needs together with development of oral microorganisms. It also results from eating, drinking, or exposure to high sugar foods which can stimulate microbial colonisation of the tooth surfaces. A cavity occurs when a tooth decays and the barrier between the saliva, and the tooth root or pulp, is breached. The inner part of the tooth contains blood vessels and a nerve. The nerve registers pain and the person feels a toothache as a result. This is caused by bacterial infections, so called dental caries.

1.2 The important dental caries causing bacteria

The human mouth contains around 500 to 1000 species of bacteria that have various functions. There are four main species within the Streptococci: these are *S.mutans*, *S.salivarius*, *S.anginosus*, and *S.mitis* groups. *S.mutans* makes up a large majority of the bacteria that affects our mouths (Marsh and Martin, 1999). Some oral bacteria act positively by producing organic acids which can help to inhibit the disease-producing microorganisms that enter via the mouth. These bacteria work with our immune system to keep our bodies relatively disease free.

The important bacterial species which are responsible for dental caries are *S.mutans* and *S.oralis*. Other species present in the mouth include *Lactobacilli*, *Actinomyces* and *Veillonella* species. *S.mutans* and *S.sobrinus* are most commonly found in humans. *S.sobrinus* is generally found in association with

S.mutans and is thought to be principally responsible for the development of smooth surface caries (Mayooran et al., 2000)

The microbial composition of dental plaque is diverse and remains relatively stable over time (microbial homeostasis). Microbial homeostasis can break down, and a major shift in the composition of the microflora can occur. For example, the frequent consumption of fermentable dietary carbohydrates is associated with an increased risk of dental caries (Marsh, 1994). Thus sugar rich diets can lead to a rise in the proportions of caries causing bacteria including *S.mutans* and *Lactobacilli*, with a concomitant decrease in the populations of other Streptococci, including *S.sanguis*, *S.oralis* and *S.mitis* (Marsh, 1994). The main location of caries is in pits and fissures and more likely to develop when food is trapped between the teeth. Thus, poor tooth hygiene, especially in terms of cleaning teeth and dental flossing on a regular basis will result in the promotion of bacteria that cause biofilms on the tooth surface and dental caries.

Dental caries is one of the most common chronic infectious diseases in the world (Anusavice, 2002; World Health Organization, 2002.). There are three major hypotheses for the etiology of dental caries: (a) the specific plaque hypothesis, (b) the non-specific plaque hypothesis, and (c) the ecological plaque hypothesis (Loesche, 1992; Marsh, 1994;Theilade, 1986). The specific plaque hypothesis proposed that only a few specific species, such as *S.mutans* and *S.sobrinus* are actively involved in the disease. On the other hand, the non-specific plaque hypothesis maintains that caries is the outcome of the overall activity of the total plaque microflora, which is comprised of many bacterial species (Theilade, 1986). The ecological plaque hypothesis suggests that caries is a result of a shift in the balance of the resident microflora which may be driven by changes in local environmental conditions (Marsh, 1994).

However, many studies indicate strongly: (1) the central role of the *mutans streptococci* in initiation of caries of smooth surfaces and fissures of crowns of teeth and suggests their potent role in induction of root surface caries; and (2)

that lactobacilli are implicated as important contributory bacteria in tooth decay, but their role in induction of lesions is not well supported. There have been studies to determine the source of infection by cariogenic bacteria. Molecular/genetic studies of the implicated bacteria isolated from humans, using randomized-blinded-interventional, and longitudinal studies indicate that *mutans streptococci* are spread vertically among humans, mostly from mothers to their children. Implications of these conclusions are briefly discussed. The most significant problems of literature interpretation include the benefits/shortcomings of salivary and plaque monitoring of the flora, the role of sugar(s) in decay as it influences the flora, and modelling strategies to predict lesion score increments as distinct from determination of the etiological role of specific bacteria. Future directions for microbiological clinical caries research are suggested, and the use of the term "caries" to describe the disease, not its lesions, has been encouraged (Tanzer et al., 2001).

Figures 1.1 and 1.2 show diagrammatically the routes and the ecological niches in which bacteria can flourish. Under normal conditions the teeth are continuously exposed and coated with saliva. Saliva is saturated with calcium and phosphate ions and capable of remineralizing the very early stages of caries formation, particularly when the fluoride ion is present. Thus, fluoride is able to slow down the progress of caries. When salivary flow is reduced or absent, there is an increase in food retention. Since the salivary buffering capacity can be lost, an acid environment is encouraged and persists for longer. This in turn encourages acidic bacteria which are able to rapidly grow under such conducive conditions and metabolize carbohydrates in the low-pH environment (Edwina, 2005).
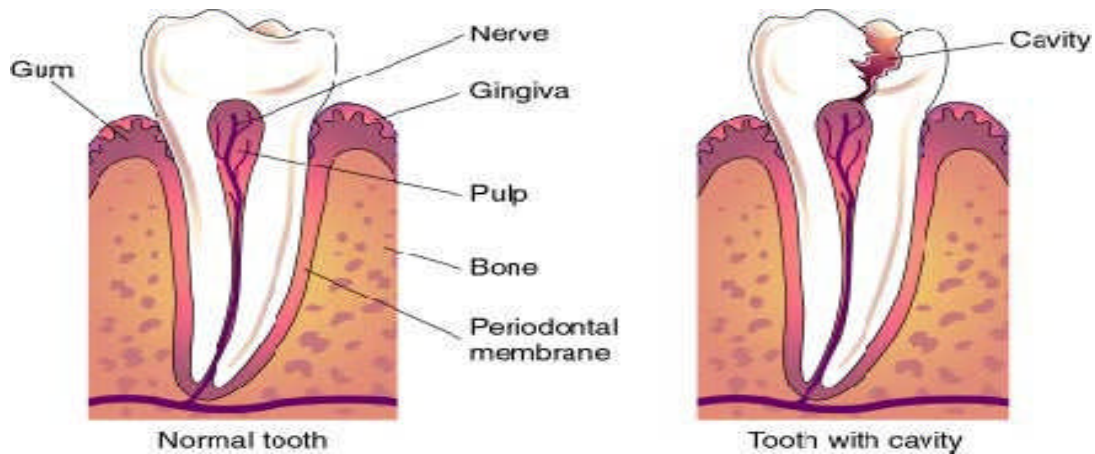
Figure 1-1: Dental caries is a disease where bacterial processes damage hard tooth structure (enamel, dentin and cementum) (Taken from zubari.rs 2009-2012)
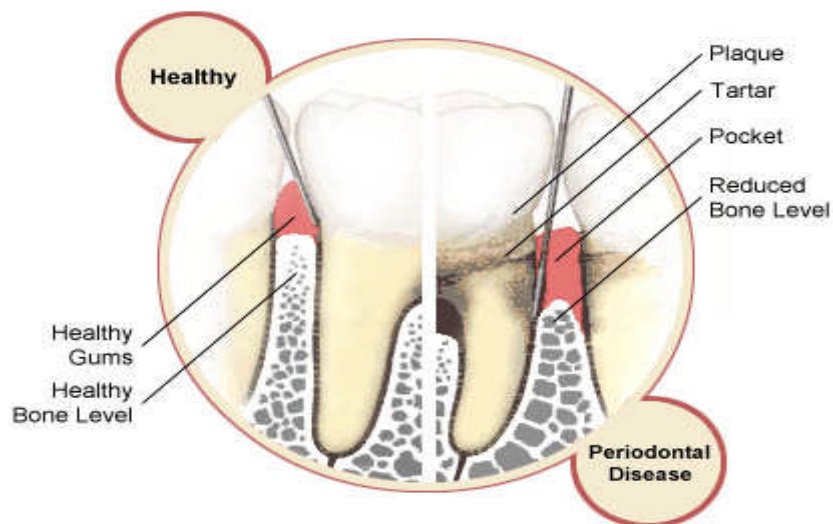
Figure 1-2: The ecological niche where oral bacteria can develop (taken from luckydentalny.com).

In children the caries process can begin within days of teeth erupting, especially if the diet is rich in carbohydrates. This can result in *S.mutans* colonising the mouths of infants, sometimes even before the teeth have erupted. They are susceptible because baby teeth have thinner enamel than permanent teeth which makes them very susceptible to caries.

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Often the transmission of *S.mutans* bacteria in infants is the result of transmission from the mother (Grönroos et al., 1998). *S.mutans* also appears capable of horizontal transmission. For example, children in the same nursery school class can often have identical strains of the bacteria in their saliva (Berkowitz, 2003). Also, children who have no detectable *S.mutans* isolated until after the age of five often share strains with both mother and father when the bacteria was finally acquired (Loveren et al., 2000). Generally, the disease process is hastened by the presence of fructose, sucrose and glucose sugars from food left on and between teeth. This is converted by the bacteria to acid and this destroys the tooth enamel, dentine and cement layers. This can result in demineralisation where enough mineral content is lost resulting in a disintegration of organic material forming a cavity in the teeth (Michael and John, 2006). They are classified by location, etiology, rate of progression, and the type of hard tissues affected.

1.3 Ecology of the dental caries causing bacteria

Ecology describes the interaction between bacteria and the structural, physical, chemical and biological components of their habitats. Infectious diseases provide examples of the impact of ecology of specific organisms on their host populations of plants or humans and other animals. Moreover, disease promotes responses from the host, changing the ecology balance between the host and the resident bacteria, influencing the well-being and activities of the host population (Locker et al., 2000).

The oral cavity provides an excellent environment for the growth and survival of bacteria. Although saliva is not a complete nutrient for all oral bacteria, some species or consortia of species utilize it as a substrate (Bowden and Li, 1997). Other oral nutrients arise from gingival crevicular fluid and desquamated mucosal cells. Also, in addition to these physiologically based nutrients, oral bacteria also have access to variable substrates from the hosts' diet, an important factor in the relative caries risk (Helderman et al., 1996). Apart from the nutritional components of saliva there are also molecules that enhance colonization and those that have an adverse effect on oral bacteria

(Scannapieco, 1994). Saliva also acts as a buffer, modifying plaque pH and reduced salivary flow. Variation in salivary flow over different tooth surfaces can influence the formation of a caries lesion (Dawes and Macpherson, 1993).

Bacteria decalcify enamel and the tooth root and may follow protein in the enamel, and invade dentine via the tubules (Thylstrup and Fejerskov, 1996). It is well accepted that the microflora of lesions in teeth and tooth roots are extremely complex and may vary at different sites (Schupbach et al., 1995). Although decalcification is a major factor involved in the initiation of enamel and root caries, degradation of dentine probably involves proteolysis (Tjaderhane et al., 1998). Also, specific receptors allow *Lactobacillus* to localize to exposed dentine, via collagen receptors (McGrady et al., 1995) and similar molecules may be present in other bacteria. These bacteria are opportunistic pathogens, found commonly as members of the resident flora of persons without caries and expressing their pathogenicity only under specific environmental conditions. *S.mutans* and *S.sobrinus*, two species of the '*mutans streptococci*' are the most significant in human caries (Bowden, 1991) and studies of the microbial ecology of caries have been directed principally at these species (Milnes and Bowden, 1985). There is also a strong association between *Lactobacillus spp.* and caries but little is known of the relative significance of the different species. In particular, although *S.mutans* and *S.sobrinus* are the principal agents of enamel caries, a wider range of organisms is proposed as opportunist pathogens in root surface caries. Generally, the organisms other than *mutans streptococci* and *Lactobacillus* associated with caries fall into Streptococcus and Actinomyces.

The ecology of the mouth does not just involve interactions among microorganisms themselves but also the host environment and teeth maturity and age of the individual. Of course, the host plays a large role in maintaining a uniform ecosystem, especially through the saliva. Saliva is a complex mineral- and protein-rich solution that delivers nutrients to the many bacterial species within the mouth while also protecting host surfaces. During mastication, increased saliva flow prevents changes in oral pH, because the buffer bicarbonate is present in saliva and acts as an acid sink at a time when acidic

products are being introduced into the mouth. Urea and the peptide saline are both also present in low concentrations in saliva and produce ammonia when hydrolyzed, a basic product capable of raising pH (Loesche, 1986). This buffering counteracts the lactic acid produced by anaerobic bacteria in the mouth during the fermentation that occurs when nutrients are introduced, offsetting decay of the teeth caused by this acid. Saliva also contains glycoproteins that are known to be antibacterial (Loesche, 1986).

1.1.1 Streptococcus mutans

Generally this bacterium inhabits the human oral cavity. It produces plaque and acids that break down tooth enamel and cause dental caries. *S.mutans* is a gram positive bacterium and is a member of the human oral flora which is widely recognized as the main etiological agent of dental caries. It has a good ability for adhesion to the tooth surface as biofilms, and it utilises glucose, fructose and lactose to produce lactic acid. The bacterium grows rapidly forming a biofilm on and around the teeth which makes them more difficult to destroy. When these dental biofilms remain on the teeth surfaces these and other acidogenic bacteria will cause the formation of cavities by the release of a range of organic acids (Lin Zhu et al., 2006). *S.mutans* can thrive in temperatures ranging from 18-40$^\text{o}$C (European Bioinformatics Institute, 2011). This species and other oral bacteria have an optimum pH in the range 6.5-7.5. Acidophilic bacteria can grow at lower pH levels (Whiley and Beighton, 1998).

It is an important bacterial species to study as it has been associated with many symptoms including tooth destruction, impaired speech, difficulty in chewing, multiple infections and has also been implicated in the pathogenesis of certain cardiovascular diseases (Nakano et al., 2006). Thus methods of control are required to minimise the ability of this bacterial species to grow.

S.mutans is one of a few specialized organisms equipped with receptors that improve adhesion to the surface of teeth. Sucrose is used by *S.mutans* to produce a sticky, extracellular, dextran-based polysaccharide that allows them to cohere, forming plaque. Molar teeth are more heavily colonized than anterior

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teeth and fissures in these teeth are more susceptible to colonization than proximal, buccal or lingual surfaces.

It has over time developed strategies to successfully colonize and maintain a dominant presence in the oral cavity. It has been able to evolve from nutrition-limiting conditions to protect itself in extreme stress conditions. Streptococci represent 20% of the oral bacteria and actually can determine the development of oral biofilms. Although *S.mutans* can be antagonized by pioneer colonizers, once they become dominant in oral biofilms, dental caries can develop and thrive.

Transmission of *S.mutans*: Like any other infectious pathogen, *S.mutans* depends on transmission routes to propagate itself among many human hosts. It favours hard, non-shedding surfaces for the establishment of permanent colonies. This led to the assumption that levels of *S.mutans* were undetectable in infants until the eruption of the primary teeth. Some studies have revealed that *S.mutans* can colonize the furrows of the tongue in pre-dentate infants (Berkowitz, 2003). When the teeth erupt, typically between the ages of one and two, and *S.mutans* can establish thriving colonies on the teeth that eventually lead to cavities, most notably Early Childhood Caries (ECC). It is the appearance of detectable levels of the bacteria on the teeth that indicate that cavity formation is possible. Detection of *S.mutans* in the furrows of the tongue reinforces the conclusion that the most common transmission route for the bacteria is vertical, from mother to child, most likely shortly after birth. Studies of saliva samples from two to five year-old children and their mothers by Caufield and Ratanapridakul (1988) and Caufield et al. (1993) revealed a high fidelity in the genetic makeup of each host's *S.mutans* population. The same study also concluded that plasmid DNA similarities correlate to different races, also implying primarily vertical transmission. As a result, mothers with high titres of the bacteria or who have suffered from dental caries themselves are likely to pass the same virulence and associated problems on to their children. In fact, mothers whose salivary *S.mutans* levels exceeded 100+ colony forming units

(CFUs) were about nine times more likely to pass the bacteria on to their children (Berkowitz, 2003).

1.1.2 Streptococcus oralis

This is a gram positive bacterium that grows characteristically in chains. It is found as an early colonizing microorganism in the oral cavity of humans and can be present in high numbers in the oral cavity. *S.oralis* grows optimally at 37°C, in both liquid films and on solid substrates. It is also able to grow under conditions of low pH, cultured at pH 5.2 or 7.

Most bacteria have an optimum pH for growth in the range 6.5 – 7.5 with limits somewhere between 5 and 9 (Whiley and Beighton, 1998).

S.oralis causes platelet aggregation and oxidation of iron in haemoglobin when it enters the blood stream via open wounds such as those created during oral surgery. And it is common cause of endocarditis and it is implicated in dental plaque formation (Marsh and Martin, 1999). *S.oralis* is the most predominant acidic non-*S.mutans* streptococcus causing significant dental caries.

S.oralis, one of commensal bacteria inhabiting the oral cavity, belongs to the oral viridans group of streptococci. It has been implicated as a potential causative organism of human cardiovascular diseases including infective endocarditis and atherosclerosis. *S.oralis* is frequently isolated from infective endocarditis (Douglas et al., 1993).

Various studies have shown that certain strains of enterococci and other oral streptococcal species including *S.sanguis*, *S.oralis*, *S.mitis* and *S.salivarius* are capable of causing caries development in rats. Formation of fissure caries, rather than smooth surface lesions, was most evident and the severity of disease was mild compared with that induced by *Mutans streptococci* (van Houte, 1980; Willcox et al., 1987). On the basis of these findings, the contribution of *non-Mutans streptococci* to the aetiology of dental caries appears minimal. The accumulated evidence from animal experiments and human epidemiological studies overwhelmingly indicates *Mutans streptococci*

are the principal aetiological agents of both enamel and root caries (Mayooran et al., 2000).

S.oralis is a numerically important member of the commensal oral microbiota, isolated from all intra-oral surfaces and a pioneer organism involved in the primary colonization of the dentition (Nyvad and Kilian, 1990). Genotyping studies using repetitive extragenic palindromic (REP)-PCR have shown that *S. oralis* is usually present as multiple genotypes in the same individual and that it is rare for unrelated individuals to share the same genotypes (Alam et al., 1999; O'Neill et al., 1999). Extensive sequencing of gdh alleles of members of the 'oralis-pneumonia-mitis' group in samples from two subjects found that the sequences clustered with the previously described species (Bek-Thomsen et al., 2008). Table 1.1 summarises the available information on effect of environmental factors on these two bacteria.

Table 1-1: Summary of the available ecological characteristics of these two bacteria

Species	Temperature range	pH	Water activity	Reference
S.mutans	Max: 30-37	6.5-7.5	0.999-0.91	EBI, 2011
S.oralis	Max: 30-37	5.2-7.0	0.999-0.91	Wiley/Beighton, 1998

In addition, the oxygen levels in the mouth may change throughout the day. These fluctuations in oral conditions can cause the populations of the biofilm within the mouth to also change because it will affect the bacteria-bacteria inter and intra-interactions.

1.4 Use of the Bioscreen approach to study microbial ecology

The rate of growth of oral bacteria will depend on the relative population present in the mouth and the availability of nutrients. The growth curves can be determined as the algorithm of the relative population size [y = in (N/N0) as a

function of time (t) (Zwietering et al., 1994)). The growth of a microbial culture at a specific temperature and under a set of environmental conditions can be followed using traditional plating methods: at specified times an amount of the test culture is transferred from the growth medium, diluted and spread plated onto a relevant nutrient agar, and incubated at an appropriate temperature (e.g. optimal for growth) for 24-48 hours or until a viable count of the colonies can be done. Plotting the resulting log numbers against the incubation time gives the standardised microbial growth curve (Figure 1.3). There are four distinct phases which can be recognised in this microbial growth curve:

Lag phase: where there is no apparent increase in the bacterial population, but the cells may be becoming conditioned and metabolically active in preparation for the next phase. The length of the lag time depends on many factors including the inoculum size, the time needed by cells to recover from any previous physical stress, e.g., environmental stresses such as low temperature, low or high pH and modified solute concentration.

Exponential (log) phase: this is when the division of the cells starts and continues at a constant rate (generation or doubling time) of the population depending on the incubation conditions (media, temperature and time) and the microbial species.

Stationary phase: when the nutrients are becoming exhausted or a lack of biological space or/and the accumulation of inhibitory metabolites; the bacterial population growth will slow down and not increase anymore. This is especially so in a batch culture with a limited nutrient content.

Death phase: during this phase the viable cell population declines and the number of viable cells decreases quite quickly (in a reverse order to the exponential (log) phase) when incubation continues beyond the stationary phase. When using a turbidimetric measurement such as the Bioscreen analyser this phase cannot be detected because the optical density and turbidity of the growth broth remains unchanged in this phase, although the number of viable cells decreases.
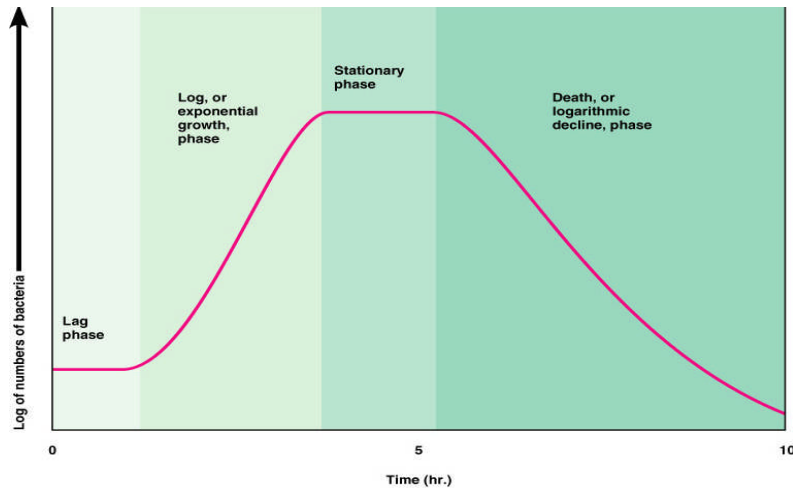
Figure 1-3: Diagrammatic representation of a bacterial growth curve with the different phases (Salih, 2010).

1.1.3 Bioscreen Microbiological Analyser

Using the traditional method for microbial growth curves is time consuming and labour intensive due to the need for multiple serial dilutions and plating of each sampling taken. Thus, to obtain one growth curve may require several days' work to obtain the data. The optical density or turbidity of a cell suspension is a non-destructive technique to determine or measure the amount of light scattered by the bacterial suspension and it is normally related to the number of cells or the mass of cells. This approach has been automated in the Bioscreen Microbiological Analyser which can be used to examine bacterial growth rates by using optical density (OD) in 100 well titre plates (100 x 2) and in which it is possible to control both temperature and agitation rates. Additionally, it is possible to measure the growth rate automatically every few seconds or minutes as required.

The optical density of microbial cells suspension increases with time as the organism grows in the medium. A plot of the $\log_{10}$ initial inoculum (cfus/ml) against time is shows in (Figure 1.4). Usually these data sets are used to determine the Time To Detection (TTD) which is usually set at 0.2 optical density value. This helps to determine the lag phase length and the relative growth rates by reference to plots of the log CFUs/ml against the TTD of different inoculum sizes.
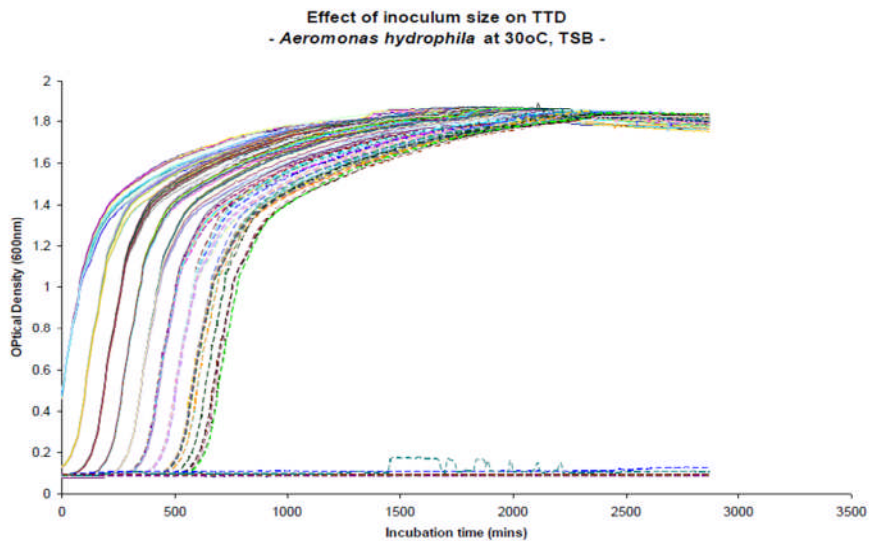
Figure 1-4: Effect of inoculums size on Time to Detection (TTD) of *Aeromonas hydrophila* at 30°C (Salih, 2010)

From this plot the time to detect an OD=0.2 for each well (curve) can be obtained and this plot of TTD against Log inoculum size can be constructed. Lambert and Pearson (2000) developed have used this approach for bacterial susceptibility testing to novel compounds. However, this depends on the medium being relatively clear as opaque media will not allow measurements to be made.

The time to detection (TTD) is defined as "the time to produce an optical density of 0.2 (Lambert and Bidlas, 2007), the assumption made that at an OD=0.2 each well in the Bioscreen plate has approx. identical numbers of microorganisms.

1.5 Control strategies

The best methods of protection from bacterial caries are the following:
- Good Oral hygiene
- The use of dental sealants as a means of prevention. A sealant is a thin plastic-like coating applied to the chewing surfaces of the molars to prevent food from being trapped inside pits and fissures
- Calcium, found in foods such as milk and green vegetables, is often recommended to protect against dental caries. It has been demonstrated

that calcium and fluoride supplements decrease the incidence of dental caries. Fluoride helps prevent decay of a tooth by binding to the hydroxyapatite crystals in enamel.

The problem is that education has often been lacking resulting in poor teeth hygiene. Thus knowledge of the fact that bacterial fermentation of dietary carbohydrates producing organic acids capable of attacking the enamel causes decay is sometimes not recognised, especially in school children. The role of the dental plaque, the sticky deposit which accumulates and adheres tenaciously to the surfaces of teeth not subjected to cleansing by mastication and the activity of the oral muscles is thus not addressed effectively.

Dental caries can be controlled first of all by personal oral hygiene care, which consist of proper brushing and flossing daily at least two times a day. The purpose of oral hygiene is to minimize any pathologic agents in the mouth. The brushing and flossing facilitates the removal and prevention of the accumulation of plaque around the teeth. Plaque consists of a mixture of bacteria and thus the plaque causing bacteria can increase, with the teeth becoming more susceptible to dental caries. The objective of regular brushing is to remove or reduce this accumulating plaque on accessible surfaces, especially proximal caries. Usually, X-rays are taken on a regular basis to monitor the potential development of cavities in high risk areas of the mouth. Chewy, sticky foods (such as dried fruit or candy) are best if eaten as part of a meal rather than as a snack. If possible, the teeth need to be brushed and rinsed with water after eating such foods. By Minimizing snacking, which creates a constant supply of acid in the mouth can be prevented the development of these dental caries causing bacteria.

Dental sealants can be used to try and prevent some cavities. Sealants are thin plastic-like coatings applied to the chewing surfaces of the molars. This coating prevents the accumulation of plaque in the deep grooves on these vulnerable surfaces. Sealants are usually applied on the teeth of children, shortly after the molars erupt. Older people may also benefit from the use of tooth sealants. It has been suggested that the chewing of xylitol-containing gum can help to

decrease bacterial growth. The bacteria cannot use the xylitol as a food source, like sugar. Other products may also reduce the acid level in the mouth.

Increased tooth resistance to caries development may be achieved by the use of fluorides. Indeed, the use of fluoride in toothpaste and other oral products is believed to be the major reason for the substantial decline in caries incidence in many developed countries (Ten Cate, 1998). Fluoride helps to prevent dental caries by binding to the hydroxyapatite crystal in enamel. Topical fluoride is also recommended to protect the surface of the teeth. This may include a fluoride toothpaste or mouthwash. Phosphates have been used as food additives to prevent dental caries. It was reported that the addition of sodium trimetaphosphates to chewing gum and calcium sucrose phosphate to the diet can prevent dental caries (Mayooran et al., 2000) and regular visits to the dentist should control the decay in the mouth.

Several previous studies found that chlorhexidine is a very effective compound with very good anti-plaque properties. In a supragingival biofilm model, chlorhexidine was shown to inhibit bacterial growth and biofilm formation (Guggenheim et al., 2001; Shapiro et al., 2002). Because chlorhexidine is positively charged, it binds to various surfaces including enamel pellicle, hydroxyapatite and mucous membranes. A major part of the effectiveness of chlorhexidine is due to this (Balakrishnan et al., 2000). However, the retention of chlorhexidine on tooth surface also leads to an undesirable side-effect which is tooth staining and calculus formation (Moshref, 2002; Yates et al., 1993). To address this problem, an oral hygiene composition comprising chlorhexidine gluconate with an anionic anticalculus agent has been suggested (Barton and Galley, 1997).

Recent advances in caries prevention using plant extracts are more focused in finding novel active extracts (Mezine. et al., 2009). They found a formulation derived from water soluble components of the Labiate family of plant extracts. This formulation was able to prevent dental plaque accumulation through inhibition of GTF enzyme activity, reduce caries-associated inflammation in the oral cavity by cyclooxygenase inhibition, and provide a strong anti-oxidative

capacity. A non-food anti-microbial-adhesion and aggregation composition comprising of a suitable carrier and an effective amount of an adhesion inhibitory fraction isolated from berry juice of the *Vaccinium* plant genus was found to be effective by Ofek et al. (2005). This adhesion inhibitory fraction was characterized as being polymeric and having a molecular weight of 14,000; an elemental analysis of carbon 43-51%, hydrogen 4-5%, no nitrogen, sulphur or chlorine. This composition was able to inhibit bacteria-bacteria interaction and interactions between bacteria and the pellicle layer on tooth surface. A possible mechanism for this inhibitory effect might be the interruption of lectin-carbohydrate interaction whereby the sugar residues on one bacterial pair interact with a lectin on the surface of the other bacterial pair (Majeed and Prakash, 2003). They also found an essential oil composition derived from *Coleus forskohlii* which showed significant inhibitory action against *S.mutans* which represents a novel natural essential oil for prevention and treatment of dental caries. There has thus been interest in finding different novel essential oils or alternative such as antioxidants which could be used to try and inhibit the growth of species such as *S.mutans* and *S.oralis*.

1.5.1 Antioxidants and essential oils

An antioxidant is a molecule that inhibits the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons or hydrogen from a substance to an oxidizing agent. Oxidation reactions can produce free radicals. In turn, these radicals can start chain reactions. When the chain reaction occurs in a cell, it can cause damage or death to the cell. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions. They do this by being oxidized themselves, so antioxidants are often reducing agents such as thiols, ascorbic acid, or polyphenols (Helmut (1997).

Antioxidants as control compounds: Antioxidants can help to maintain the balance between oxidative stress and other oxidation reactions. There are several thousand antioxidants, including enzymes, vitamins, minerals and other nutrients and compounds. Some antioxidants are produced within the body;

others, such as vitamins A and C, must be provided by external sources. A healthy, varied diet rich in fruits and vegetables, whole grains and nuts is an excellent source of antioxidants. Antioxidants may be supplied by other external means as well.

Antioxidants have been commonly examined for efficacy against microorganisms involved in disease as well as in food applications. The esters of p-hydroxy benzoic acid (paraben) were found to be very effective in inhibiting growth of spoilage bacteria and fungi. There mode of action may be at the cell membrane level eliminating the pH-related component of the protomotive force and affecting energy transduction and substrate transport. BHA has also been shown to have a direct effect on the mitochondrial electron chain of trypanosomes, thus inhibiting respiration. Antioxidants have also been found to be effective in treatment of disorders associated with gingival tissues and other supporting structures of the teeth (San Miguel et al., 2011).

Essential oil: these are natural extracts which have been examined as new natural antimicrobial therapeutic agents for control of microbial diseases. They have been investigated for the control of many bacterial species including dental caries causing bacteria such as *S. mutans*. They are complex, volatile, natural compounds formed by aromatic plants as secondary metabolites. They are known for their bactericidal, virucidal, fungicidal, sedative, anti-inflammatory, analgesic, spasmolytic, and locally anesthetic properties. The presence of complex chemical structures constituted of several groups, such as terpenes and terpenoids, aromatic and aliphatic constituents, all characterized by low molecular weight, may explain their successful bacteriostatic and bactericidal action. Detailed studies of essential oils for efficacy against the two Streptococci examined in this thesis are however limited (Lıvia Camara et al., 2012).

1.6 Aims and Objectives

The aims of this study was to examine two dental caries causing bacteria, *S.mutans* and *S.oralis,* as model systems to examine the efficacy of using antioxidants and essential oil components to inhibit their growth. The second

aim was to examine whether the best ones could be combined with fluoride for better efficacy. The third aim was to provide more ecological data on the activity of these two bacteria under different environmental conditions (temperature, water activity).

1.6.1 The main objectives of this work were:

1. An initial screening of nine essential oils and three antioxidants on growth of *S.mutans* and *S.oralis* was done using a clearing zone assay at 37°C.

2. Examination of the effect of different concentrations of the best essential oil and antioxidant treatments on the numbers of viable CFUs of *S.mutans* and *S.oralis* after incubation at 37°C for 24-48hrs.

3. Determination of the ED_{50} and ED_{90} concentrations of the best compounds for inhibition of these two dental caries bacteria

4. To examine the potential of combining the best compounds in combination with fluoride for improved control of these two bacterial carries causing organisms in defined media and in an artificial saliva medium

5. Ecological studies to examine the effect of pH and water activity on growth of these two Streptococci species using the Bioscreen instrument.

2 MATERIALS AND METHODS

2.1 Bacterial strains used in this study

A type culture of *S.mutans* (11516) and *S.oralis* (702680) were obtained from the UK National Culture Collection in Scotland. .These were cultured as per instructions and sub-cultured regularly on Tryptone Soya Broth (TSB) and on Tryptone Soya Agar 9 cm Petri plates and kept at 4^oC until required.

2.2 Media, essential oils and antioxidants

Two types of media were used in this study. These included TSA (tryptone soya agar) and TSB (tryptone soya broth).

For the preparation of TSA, 40 grams of TSA was weighed into 1 L of water and the mixture shaken well before autoclaving at 120^oC for 25-20 mins. The molten cooled agar was poured into 9 cm Petri plates (approx. 15 ml per plate). These were stored at 4^oC until used.

TSB was prepared by weighing 30 g of the medium in 1 L of water. This was well mixed and heated. The medium was dispensed into 20 ml Universal bottles (10 mls) and autoclaved as detailed previously. These were also stored at 4^oC until used in experiments.

Overall, initial studies showed that both *S.mutans* and *S.oralis* grew well on both TSA and TSB when incubated at 37^oC for 24-96 hrs.

2.3 Essential oils and antioxidants used in this study

An initial screening was done using the following list of essential oils obtained from (F.D. Copeland & Sons, Ltd., London):

- Oil of clove leaf
- Oil of spearmint
- Oil of thyme

- Oil of lemongrass

- Oil of mandarin

- Oil of sweet fennel

- Oil of ginger

- Oil of clove bud

- Oil of cinnamon leaf

These essential oils (Sigma Aldrich, UK) were diluted in methanol (1g of essential oil to 10ml of methanol).

The antioxidants examined in this study were:
- Butylated hydroxy toluene (BHT)

- Butylated hydroxy anisole (BHA)

- Propyl gallate   (PG)

1 gram of the antioxidant was added to 10 ml of methanol, thus making up a 10% concentration solution of each one.

2.4 Initial screening of essential oils and antioxidants

A traditional clearing zone screening assay was used to compare the efficacy of the 10% concentrations of the essential oils and antioxidants for obtaining the most inhibitory treatments.

The agar media were inoculated with a 200 ul of each bacterial species (*S.mutans*; *S.oralis*) and spread using a sterile glass spreader over the whole agar plate. Then, three holes (5 mm diam, with a sterile cork borer) were made equidistant from each other on replicate Petri plate treatment. The experiments were carried out in duplicate. In each of these holes a 25 ul of the diluted essential oil and one hole was filled with methanol as a control. The treatments and replicates were incubated at 37°C for 48 hrs. After 24 and 48 hrs, the zones of clearing around the essential oil treatments were examined and the diameter (mms) measured. These experiments were repeated twice to confirm the results.

Similar methods were used for the testing of the antioxidants. The bacteria were spread plated onto the media and then the 25 µl of the test antioxidants were added to the wells made in the agar plates as detailed previously. The treatments and replicates were again incubated at 37°C and the clearing zones measured after 24 and 48 hrs.

2.5 Testing of best treatments for determining ED_{50} and ED_{90} values for control of S.mutans and S.oralis

Screening of essential oils and antioxidants: For these experiments clove leaf, cinnamon oils and BHA, PG antioxidants were tested. For essential oils the following concentrations were tested: 0.1, 1, 5 and 10%. These were added in methanol to the TSB media.

Similarly BHA and PG were also added to the liquid broth media.

Three replicates of each treatment including controls were incubated at 37°C for 24 hrs. Then 100 µl of each treatment and replicate were spread plated onto TSA agar plates (3 replicates per dilution) and the numbers of viable colonies counted at each concentration for each treatment and replicate. The viable populations were compared with the controls. This enabled the ED_{50} and ED_{90} concentrations to be quantified by reference to the control populations..

Fluoride solutions and ED_{50} and ED_{90} concentrations: For these experiments sodium fluoride stock solutions in sterile water was initially made up (10.000ppm). Concentrations of 100, 500 and 1000 ppm were used as the treatments concentrations. The effect of treatments was investigated as described previously and after incubation at 37°C for 24 hrs. An inoculum of 100 µl of a 10^{4} CFUs/ml concentration was used in these studies.

Experiment of antioxidants or essential oils + sodium fluoride: For these experiments 0.5% and 1% of essential oils and antioxidants were used in combination with 1000 ppm of NaF (filter sterilised through a 0.22 micron sterile filter. In this case the essential oils/antioxidants were dissolved in methanol and then added to 10 ml TSB media in combination with 1000 ppm NaF in 25 ml Universal bottles.

The TSB treatments and replicates were inoculated with 100 µl of a 24 hrs culture (10^4 CFUs/ml) of either *S.mutans* or *S.oralis*. These were incubated for 24hrs at 37^oC. Subsequently, 12ul of each treatment for each species was spread plated onto three replicates TSA plates including the controls and incubated for 48 hrs at 37^oC to examine the viability of the treatments and replicates.

2.5.1 Artificial saliva media and efficacy of the best treatments

Studies were subsequently carried out with an artificial saliva medium to examine under more realistic conditions the effect of the best treatments in the presence of NaF. The artificial saliva medium consisted of NaCl, 0.4 g; KCl, 0.4 g; $CaCl_2.2H_2O$, 0.795 g; NaH_2PO_4 $2H_2O$, 0.78 g; NH_2CONH_2, 1.0 g; distilled water, 1000 mL The pH of the medium was 3.5.

For efficacy of NaF on growth of the two bacteria the concentrations of 100, 500 and 1000 ppm were tested as described previously using the initial stock solution of 10,000 ppm.

Studies were then carried out with the antioxidants and essential oils (0.5 and 1% concentration) and sodium fluoride (1000 ppm) on the viability of *S.mutans* and *S.oralis* by incubation in the artificial saliva medium for 24 and 48 hrs and then checking viability by plating on TSA medium. In all cases the experiments were carried out in duplicate and repeated twice.

2.6 Ecological studies using the Bioscreen system

There is little detailed information on the effect of pH and water activity on the growth of *S.mutans* and *S.oralis*. This study utilised the Bioscreen method to examine the effect of different pH values (4-7) and ionic solute concentrations (1-6% NaCl= 0.999, 0.99, 0.98, 0.97 water activity) on the growth of these two bacteria by comparing the TTD under different ecological conditions in TSB. The Bioscreen uses the automated optical density (OD) measurements to effectively monitor and measure the growth of bacteria in real time (Begot et al., 1996).

2.6.1 Bioscreen system

The Bioscreen machine is an automated turbidity reader which uses 2 x 100 well micro titre plates, linked to an integrated PC (Lab systems, Helsinki, Finland). The temperature can be accurately controlled and provides growth curves from each well directly and the data sets based on monitoring on a very regular basis (5-10 secs to 5-10 mins) can be downloaded and analysed using other software (in this case Excel was used).

2.6.2 Culture preparation and growth studies in the Bioscreen

Bacteria were grown overnight in conical flasks containing 80 ml TSB in shaken cultures at 37°C. The cells were harvested, centrifuged at 3000 rpm (10 min) and the resulting cell pellets resuspended in 2 ml TSB. The inoculum was standardised by diluting to an approximate OD = 0.5 at 600 nm giving approximately 2×10^5 cfu/ml. This standardised culture was subject to either ten decimal or ten half –fold dilutions in TSB.

Each well in the Bioscreen microarray plates was filled as follow:

200ul of TSB was decanted into each of the wells except column 10.

- ➢ The wells of column 10 were filled with 400µl of the appropriate serial dilutions (decimal or half fold), with the highest inoculum (the zero dilution) in well 100

- ➢ Using a multi-pipette, 200µl were removed from each well of column 10 and transferred into the wells of column 9, mixed by repeated syringing, and then 200µl were removed (using new tips) from the wells of column 9 and transferred to column 8 etc. This was repeated across the line finishing with column 1 (discarding 200µl after final mixing). There was no need for a negative control as this is the background OD of the broth

Extra care was taken when performing repeated syringing to get the right dilution and to avoid carrying extra cells between dilutions. This was done by changing the tips of the multi-pipette for each column and also by placing the

tips in at the right depth of each well (if the tips are not placed far enough into the well, bubbles form which impaired the performance of the experiment.

Plating and colony counting: From the tubes labelled -5 and -6 decimal dilution, 0.1 ml of each was transferred and spread onto previously prepared TSA plates in triplicate and incubated at 37℃ for 1-2 days. Plates with <300 CFUs were counted and the approximate log number of the initial (zero dilution) culture were calculated. The following calculation is an example of this method:

- ➢ Plates counts for the -6 dilution: 102, 123 and 107 colonies

- ➢ Average counts: 111 colonies

- ➢ Due to the plating dilution the number of colonies are multiplied by 10 (111x10)

- ➢ To get the approximate colony number in the Zero dilution multiply by 106 (6 serial dilutions from -6 to 0)

- ➢ The initial inoculum was: 1.11×10^9 cfus/ml.

An example of the temporal effect of different concentrations of initial inoculum on growth rates and optical density of *S.mutans* and *S.oralis* is shown in Appendix I.

2.6.3 Effect of pH and water activity on growth of *S.mutans* and *S.oralis* using the Bioscreen

The pH of the media was modified using buffers as described below using phosphate/citrate buffers. The amounts of each component are detailed below to obtain the target pH values.

1. pH 4: 19.3ml (Na_2HPO_4) + 30.7ml (citrate)

2. pH 5: 25.7ml (Na_2HPO_4) + 24.3ml (citrate)

3. pH 6: 32.1ml (Na_2HPO_4) +17.9 ml (citrate)

4. pH 7: 43.6ml (Na_2HPO_4) + 6.5ml (citrate)

pH narrow range indicator strips were used to check the accuracy of the treatments. These were confirmed by using a pH meter and shown to be accurate.

In the experiments the wells were filled with 150ul of pH treatment to a column of 8 wells with two replicates per treatment. This gave a total of 16 wells per treatment. To this 50 ul of the bacterial suspension (at the same pH, x CFUs/ ml) added. The last two wells in each column were filled with the control TSB solution (200 µl). This was repeated for each of the pH levels tested with two x 8 column of wells for each treatment. The plates were incubated in the Bioscreen machine at 37°C for 24-48 hrs.

The effect of water activity was determined by examining the effect of different ionic solute concentrations on growth of *S.mutans* and *S.oralis*. The concentrations used in TSB were 1, 2, 4 and 6% NaCl (=0.999, 0.99, 0.98 and 0.97 water activity).

150 µl of NaCl treatment was added to each well in two columns of 8 wells. To this 50 ul of the bacterial suspension (at the same NaCl, x CFUs/ml) added. The first two wells in each column were filled with the control TSB solution (200 µl). This was repeated for each treatment. The plates were incubated in the Bioscreen machine at 37°C for 24-48 hrs.

The data was plotted and the time to detection (TTD) for each treatment was compared with a standard initial inoculum in the ecological studies.

2.6.4 Statistical treatment of results

The means of three replicates were made in the screening assays, Analysis of variance was used to examine the relationship between treatments in the assays examining viability assay of individual and treatments combined with fluoride and the treatments which were significant at $P=0.05$ identified.

3 RESULTS

3.1 Screening of essential oils

S.oralis:

Figure 3.1 show the effects of the 9 essential oils on growth of *S. oralis* after 24 and 48 hrs. The clove leaf, thyme and cinnamon oils were found to be effective after 24hrs. For the others, including clove leaf, thyme, cinnamon, ginger, mandarina and clove bud they were only effective after 48hrs incubation.
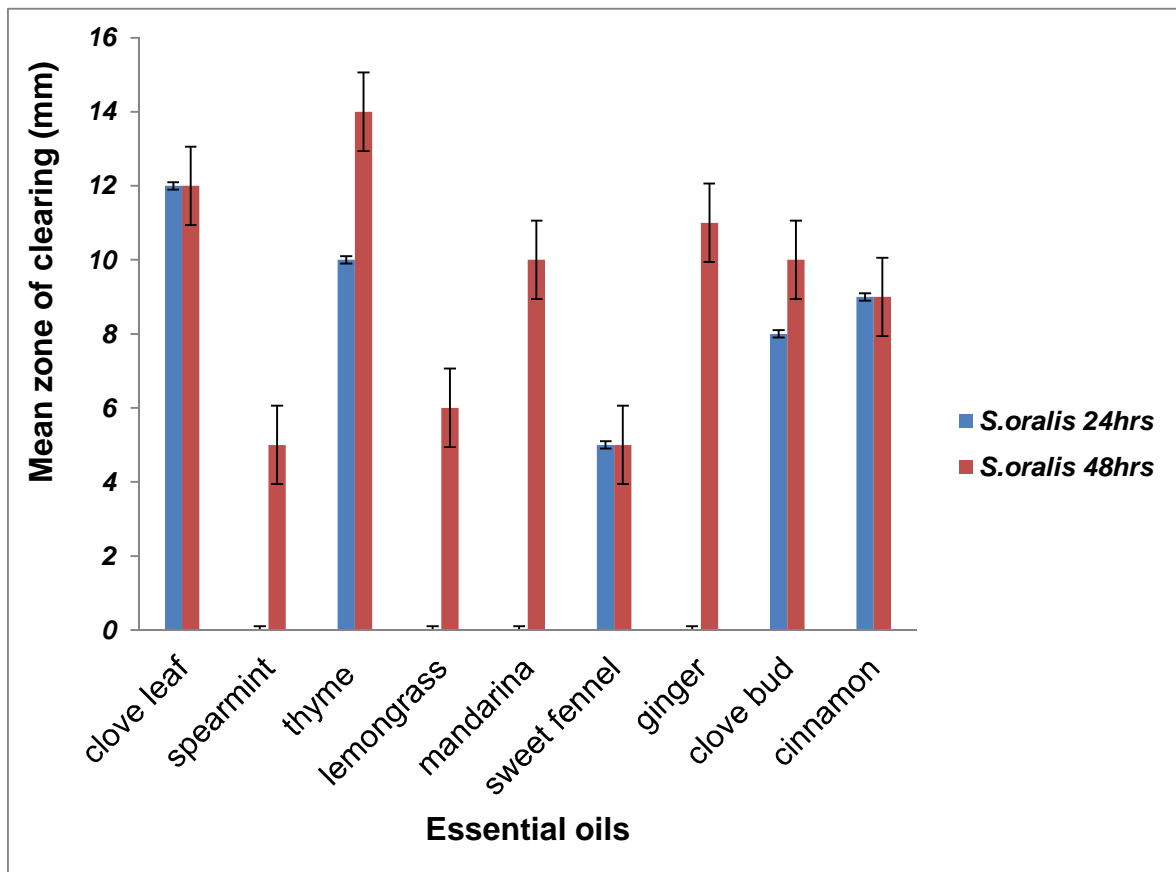
Figure 3-1. The effect of 9 essential oils on growth of *Streptococcus oralis* after 24 and 48 hrs using two types of media (TSA, TSB) and the concentration of each treatment was 10%.

S.mutans:

Figures 3.2 the effect of the 9 essential oils on growth of *S.mutans* after both 24 and 48 hrs incubation. For this species, clove leaf, cinnamon and clove bud oils were found to be effective in inhibiting growth after 24 hrs. The other treatments were ineffective after 24 hrs incubation. Lemongrass, mandarin and ginger essential oils only had some effect after 48 hrs.
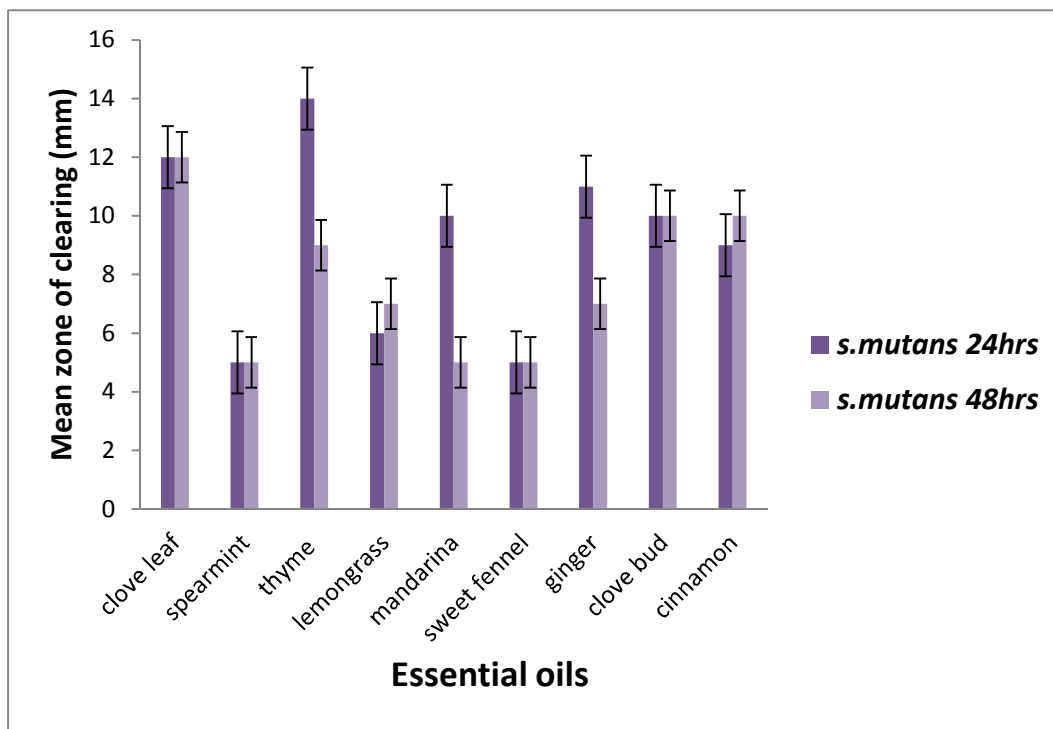
Figure 3-2The effect of 9 essential oils on growth of *Streptococcus mutans* after 24 and 48 hrs using two types of media (TSA, TSB) and the concentration solution of each was 10%. Bars indicate SE of the means.

3.2 Screening of antioxidants

Figures 3.3 shows the effect of 3 antioxidants tested on control of *S.oralis* colony growth after 24 and 48 hrs. The BHA and PG were found to be the most effective treatments. Figure 3.4 show that for *S.mutans* similar effects were observed, with the same two antioxidants being more effective than BHT.
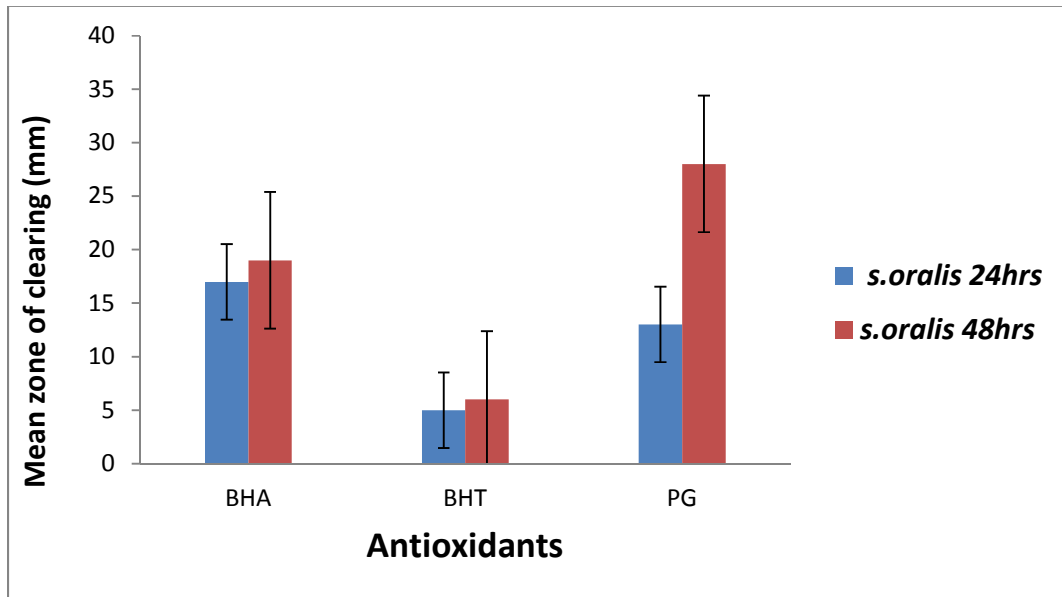
Figure 3-3 Effect of three antioxidant treatments on the mean clearing zones for control of *S.oralis* at 37°C after 24 hrs. Key to antioxidants: BHA, Butylated hydroxy anisole; BHT, Butylated hydroxy toluene; PG, Propyl gallate using two types of media (TSA, TSB) using a concentration of 10%. Bars are SE of the means.

S.mutans:
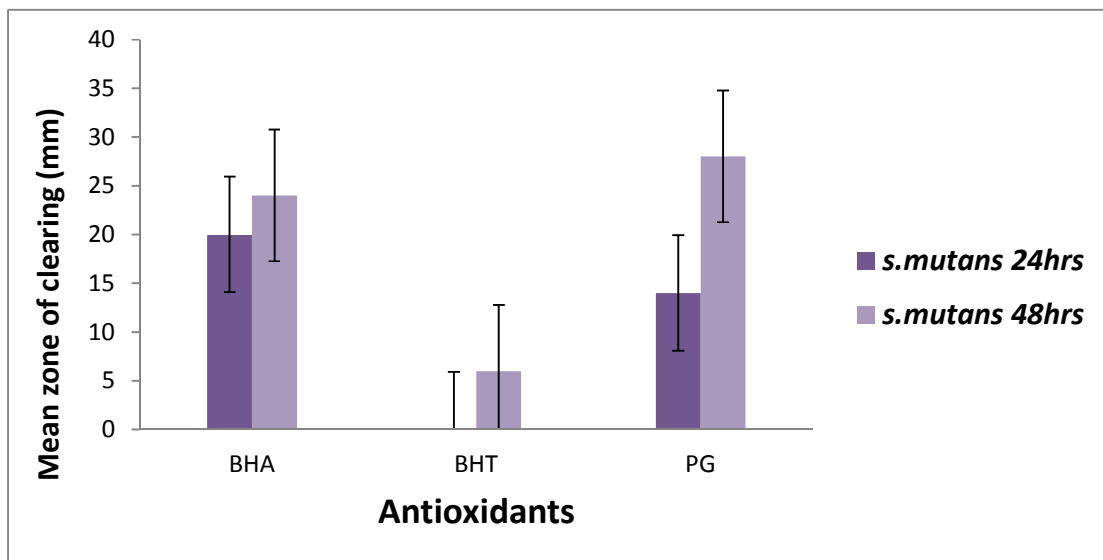
Figure 3-4 Effect of three antioxidants on inhibition of growth of *S.mutans* after 24hrs incubation at 37°C. Key to antioxidants: BHA, butyl hydroxy anisole; BHT, butylhydroxytoluene; PG, propyl gallate using two types of media (TSA, TSB) and a concentration of 10%. Bars indicate SE of the mean.

Overall, based on the initial screening experiments, it was demonstrated that clove leaf and cinnamon oils both gave the best results in terms of inhibition of the two bacterial species. Of the antioxidants tested, BHA and PG appeared to be the best treatments for further testing.

3.3 Determination of efficacy of best compounds and ED_{50} and ED_{90} values

Figure 3.5 and 3.6 show the effect of different concentrations of best two essential oils and two antioxidants on the viability of cells of both *S.oralis* and *S.mutans*. This shows that for the former species the propyl gallate was the most effective with complete inhibition by 5% concentration.

For *S.mutans*, only the propyl gallate treatment at 10% was able to completely inhibit growth of this dental caries species. Against this species clove oil was effective but did not inhibit viability completely at any of the concentrations tested.

Based on these results the approx. concentrations of the treatments required for 50 and 90% inhibition of viability were calculated and are shown in Table 3.1 and 3.2.
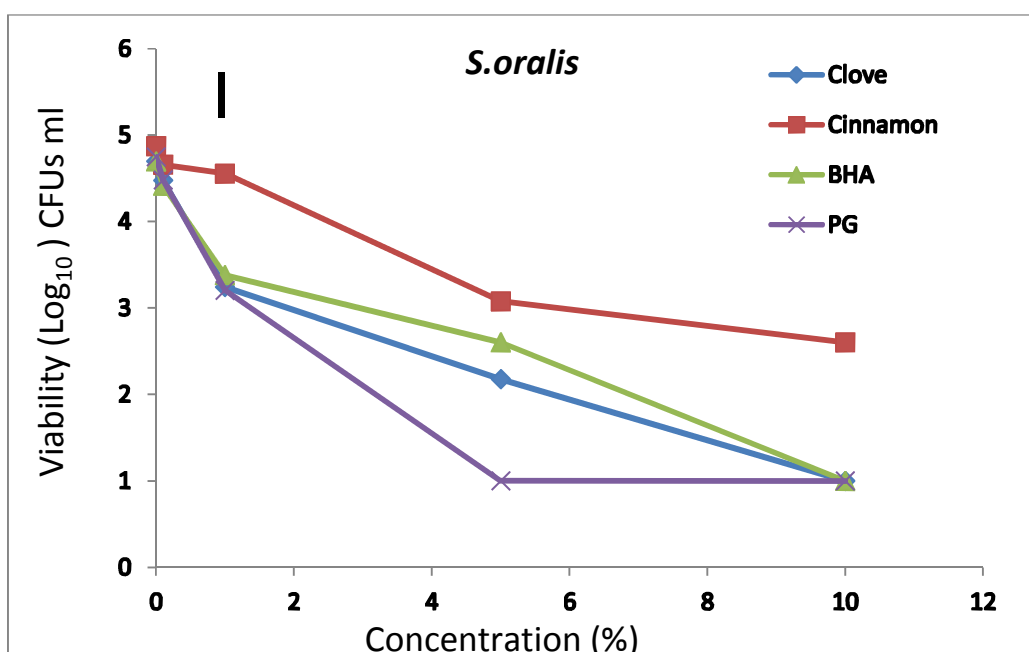
Figure 3-5 Effect of the four treatments on the relative viability of *S.oralis* cells 48 hrs after treatment by plating onto TSA. These are the means of three replicates. Bar indicates Least Significant Difference (P=0.05).
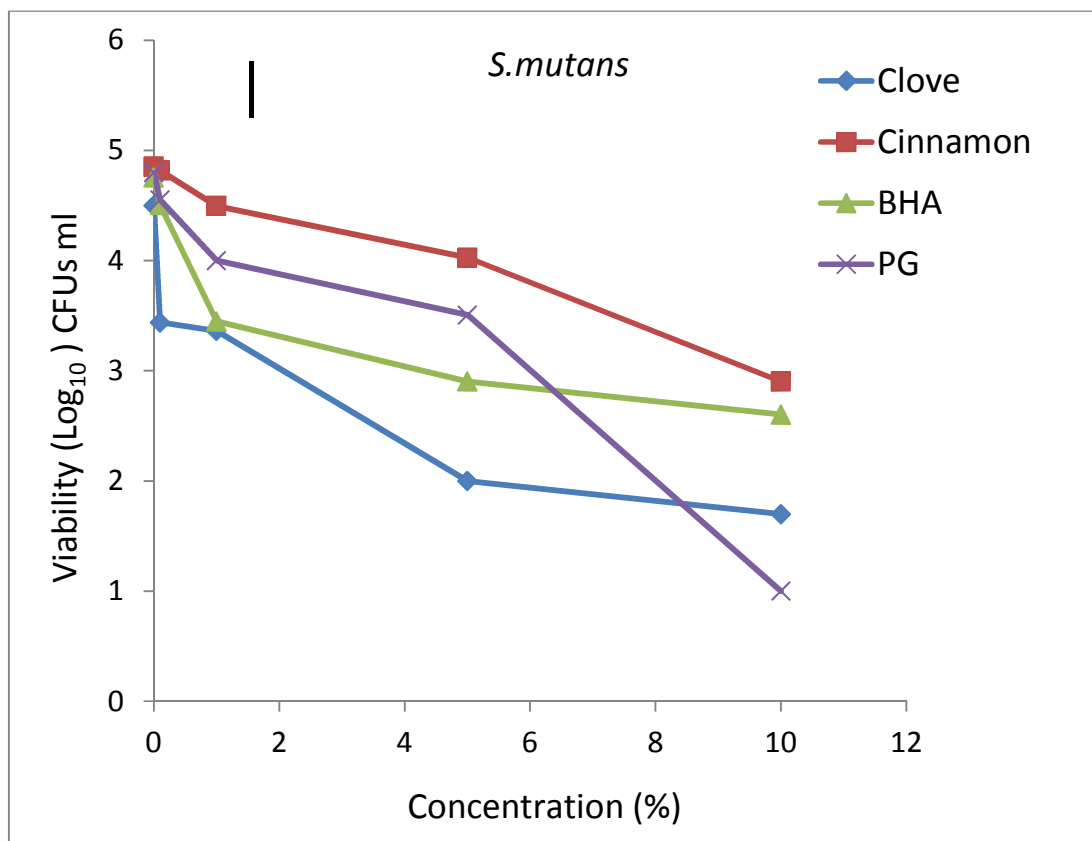
Figure 3-6 The effect of the four treatments on the relative viability of cells of _S.mutans_ 48 hrs after treatment by plating on TSA. The data are means of three replicates per treatment. Bar indicates Least Significant Difference (P=0.05).

Table 3-1. Calculated ED$_{50}$ (%) and ED$_{90}$ values based on colony viability in different concentrations of the treatments against _S.oralis_

	ED$_{50}$	ED$_{90}$
Clove leaf	4.2	<10
Cinnamon oil	>10	>10
BHA	6	9.8
PG	2	4.6

Table 3-2. Calculated ED_{50} and ED_{90} (%) values based on colony viability in different concentrations of the treatments against *S.mutans*

	ED_{50}	ED_{90}
Clove leaf	**4**	**>10**
Cinnamon oil	**>10**	**>10**
BHA	**>10**	**>10**
PG	**6.9**	**9.6**

3.4 Effect of sodium fluoride concentrations on growth of *S.mutans* and *S.oralis*

(a) S.mutans

Figure 3.7 shows the growth of *S.mutans* in sodium fluoride at different concentrations at 37°C for 24hrs. This shows that at 500 and 1000 ppm there was a >75% reduction in colonies of this bacteria.
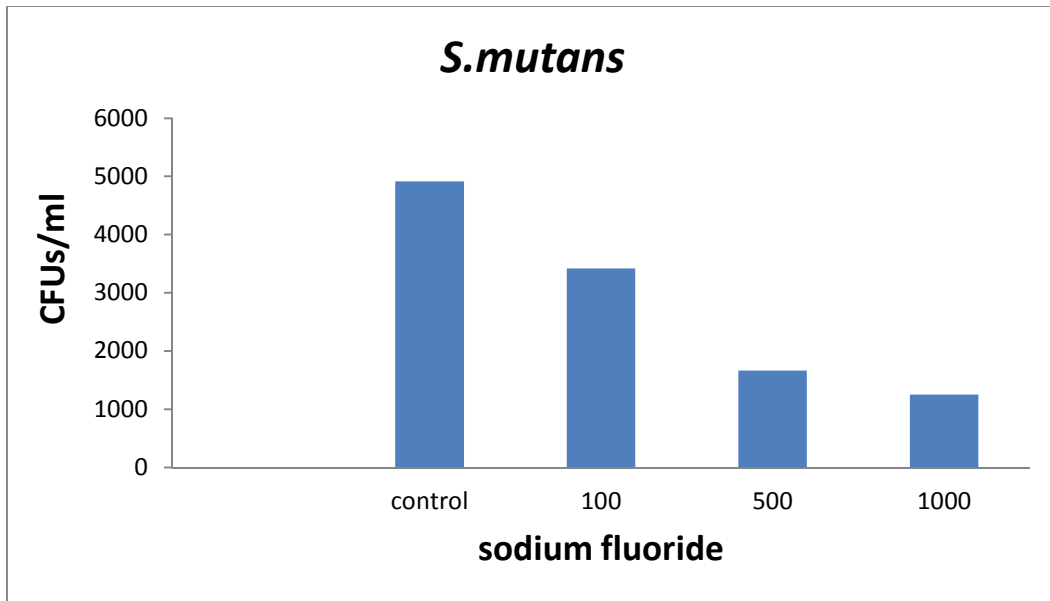
S.mutans

Figure 3-7 Effect sodium fluoride concentrations on populations of *S.mutans* in relation to concentrations of sodium fluoride at 37°C for 24 hrs. Data are means of three replicates per treatment.

(b) *S.oralis*

Figure 3.8 shows the effect of sodium fluoride on populations of *S.oralis* at 37°C for 24hrs. The efficacy of sodium fluoride against this bacterium was not as effective with only approx. 25-30% reduction in populations.
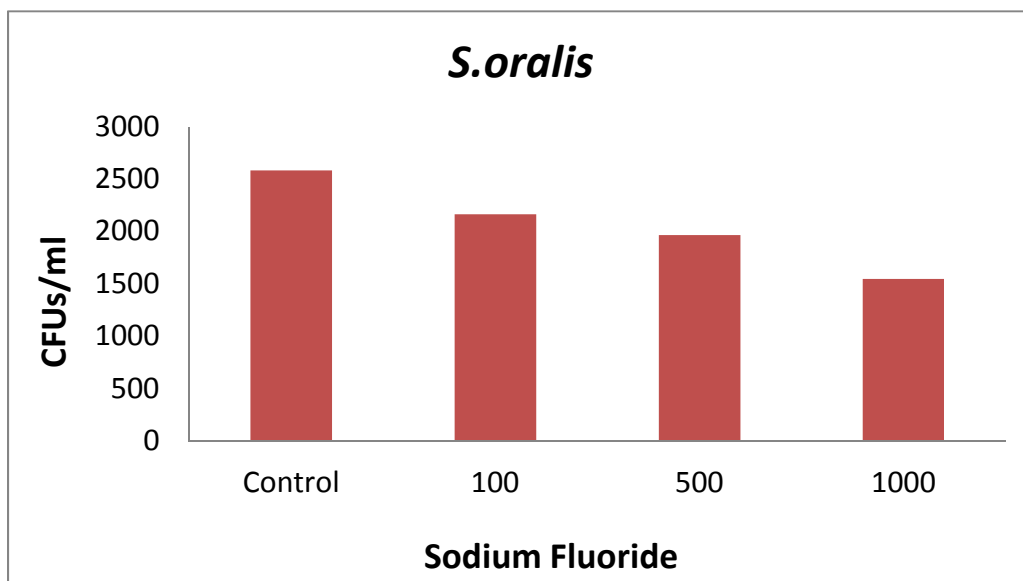
S.oralis

Figure 3-8 Effect sodium fluoride concentrations on populations of *S.oralis* grown at 37°C for 24 hrs. Data are means of three replicates per treatment.

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3.5 Effect of mixtures of antioxidants and essential oils with sodium fluoride on control of the two bacteria in TSB medium

Table 3.3 and 3.4 shows the effect of mixtures of anti-oxidants/essential oils (0.5%, 1% concentration) with sodium fluoride (2000 ppm) on the growth of the two bacteria. This shows that all treatment completely inhibited growth when compared with the untreated controls regardless of the combinations used.

Table 3-3. Effect of combinations of antioxidants or essential oils at 0.5% concentration when combined with 2000 ppm of sodium fluoride (NaF). Mean of three replicates per treatment. Key to treatments: BHA, butyl hydroxyanisole; PG, propyl gallate In TSB medium. All treatments significantly reduced viability of cells.

	CFUs/ml	
Treatment	*S.mutans*	*S.oralis*
Control	3.96	3.92
BHA + NaF	1	1
PG + NaF	1	1
Cinnamon + NaF	1	1
Clove leaf + NaF	1	1

Table 3-4. Effect of combinations of antioxidants or essential oils at 1% concentration on viable populations (Log$_{10}$ CFUs/ml) when combined with 2000 ppm of sodium fluoride (NaF) in TSB medium. Means of three replicates per treatment. Key to treatments: BHA, butyl hydroxyanisole; PG, propyl gallate.

	CFUs/ml	
Treatment	*S.mutans*	*S.oralis*
Control	3.48	3.86
BHA + NaF	1	1
PG + NaF	1	1
Cinnamon + NaF	1	1
Clove leaf + NaF	1	1

3.6 Effect of treatments with sodium fluoride on efficacy in an artificial saliva medium

Effect of sodium fluoride on growth of the two bacteria: Table 3.5 shows the effect of different concentrations of sodium fluoride alone on populations of the two bacteria after 24 hrs. in an artificial saliva medium at 37°C. This shows that the treatment was only effective against both bacteria at 1000 ppm. However, at this concentration both bacteria could still grow. Thus a higher concentration would be required to inhibit growth effectively.

Table 3-5. Efficacy of sodium fluoride on Log$_{10}$ CFUS/ml populations of *S.mutans* and *S.oralis* after 24 hrs. in artificial saliva medium treatment at 37°C. Means are of three replicates per treatment. *, significant difference from the control at P=0.05.

	CFUs/ml	
Treatment	*S.mutans*	*S.oralis*
Control	3.39	3.53
100ppm	3.30	3.21
500 ppm	3.27	3.04*
1000 ppm	3.20*	2.96*

3.7 Effect of mixtures of antioxidants and essential oils with sodium fluoride on control of the two bacteria in artificial saliva medium

Figure 3.9 shows the effect of 0.5% antioxidants and essential oils + sodium fluoride in artificial saliva on growth *S.mutans* and *S.oralis* at 37°C for 24hrs. This shows that for both species a >95% reduction in viable colonies was obtained by combining the best treatments with sodium fluoride.
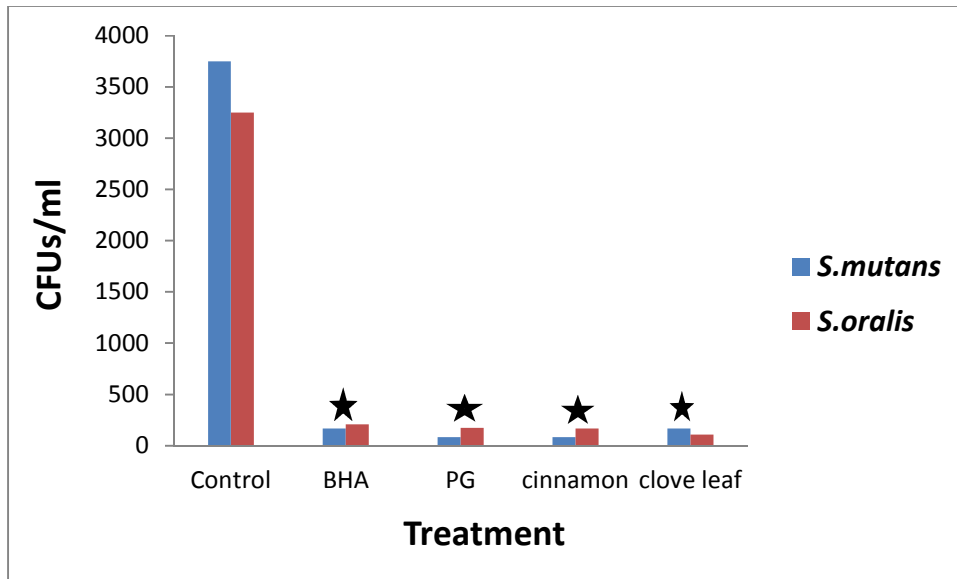
Figure 3-9 Growth _S.mutans_ and _S.oralis_ in 0.5% of antioxidants and essential oils + sodium fluoride in an artificial saliva medium after 24 hrs incubation. Key to treatments: BHA, butyl hydroxyanisole; PG, propyl gallate. Asterisk indicates significant inhibition of both species at P=0.05.

Figure 3.10 shows the efficacy of 1% of antioxidants or essential oils + sodium fluoride in artificial saliva medium on the viable populations of _S.mutans_ and _S.oralis_ after 24 hrs incubation at $37^{o}C$ and then plated onto TSB medium. There was a significant reduction in the viable populations especially of _S.mutans_. The efficacy of combined treatments was less effective against _S.oralis_. All combined treatments significantly inhibited the two species.
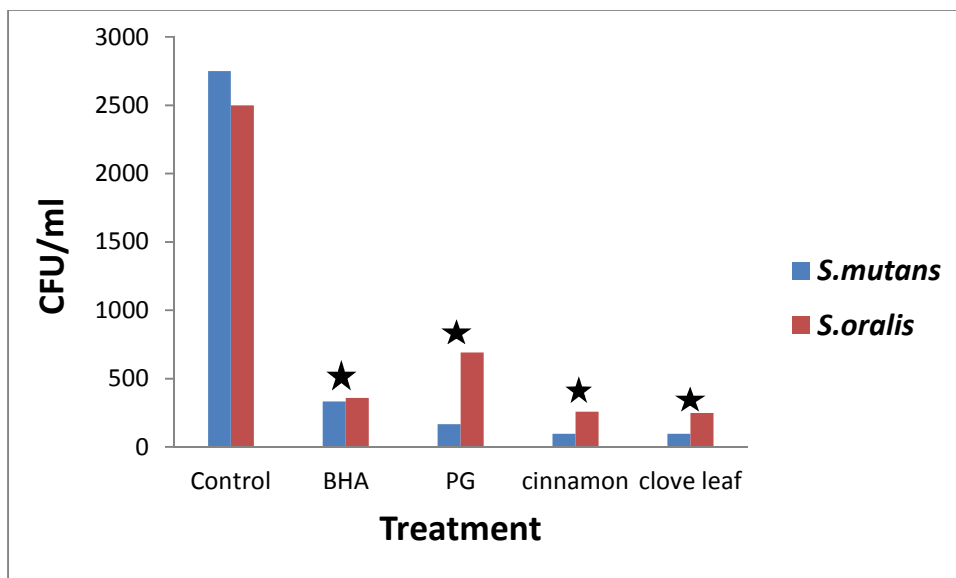
Figure 3-10 Effect of 1% antioxidants or essential oils + sodium flouride on viablility of cells of *S.mutans* and *S.oralis* incubated in an artificial saliva medium for 24 hrs. Key to treatments: BHA, butyl hydroxyanisole; PG, propyl gallate. Asterisks show significant reduction of both species (P=0.05).

3.8 Effect of pH and NaCl concentrations on growth of *S.mutans* and *S.oralis* using the Bioscreen system

Figure 3.11 shows the example of the effect of different pH levels on the growth of *S.mutans* at pH 4, 5, 6 and 7. It is clear that growth of the 10 x 2 replicates of pH 7 showed the most rapid growth, followed by pH 6 and 5. At pH 4 growth was the slowest and there was a much longer delay before growth was initiated. The replicates also show a greater variation at this pH . The Time To Detection (TTD) values were calculated and these were found to change as the pH was made more acidic. This is a good indicator of the effect of pH on the growth of both *S.mutans* and *S.oralis* (Table 3.9).
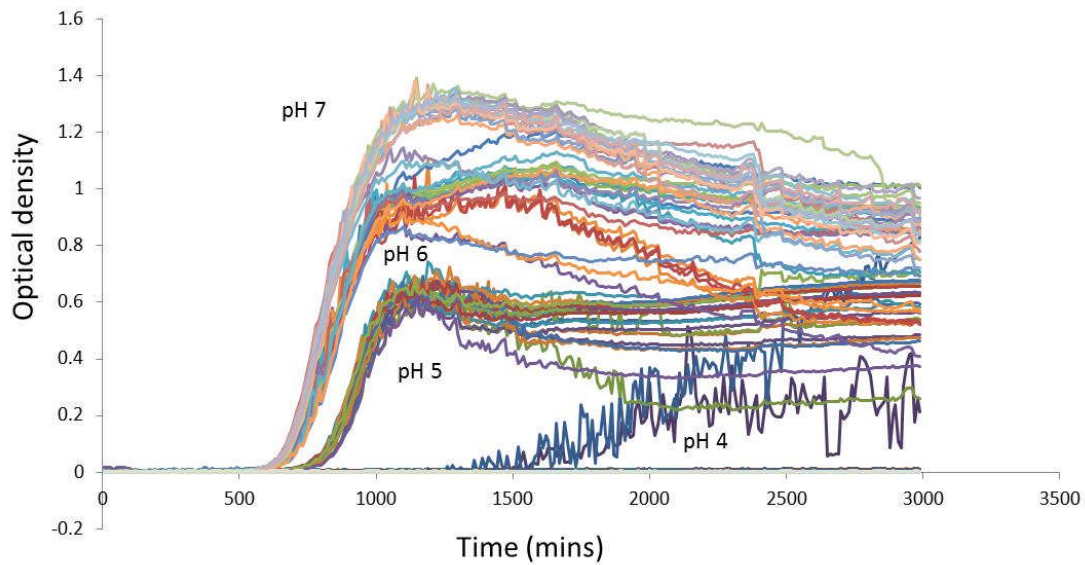
Figure 3-11Effect of pH 4-7 on relative growth of *S.mutans* over periods of 3000 mins on a TSB medium at 37°C. Lines represent wells for each replicate of each pH treatment.

Table 3-6 The relative Time To Detection (mins) for the effect of pH on *S.mutans* and <u>*S.oralis*</u> based on time required to reach 0.2 optical density.

pH	*S.mutans*	*S.oralis*
7	666.5	695.61
6	719.31	703.45
5	749.05	812.36
4	>1000	>1000

Figure 3-12 and Figure 3-13 show the effects of different NaCl concentrations on the growth of *S.mutans* and *S.oralis* on TSB at 37°C. This shows that as the percentage NaCl was increased from 1-2% to 6% (0.999 to 0.97 water activity)

the growth was delayed and the rate of growth was slower over the experimental period. The lag times prior to growth were also increased.
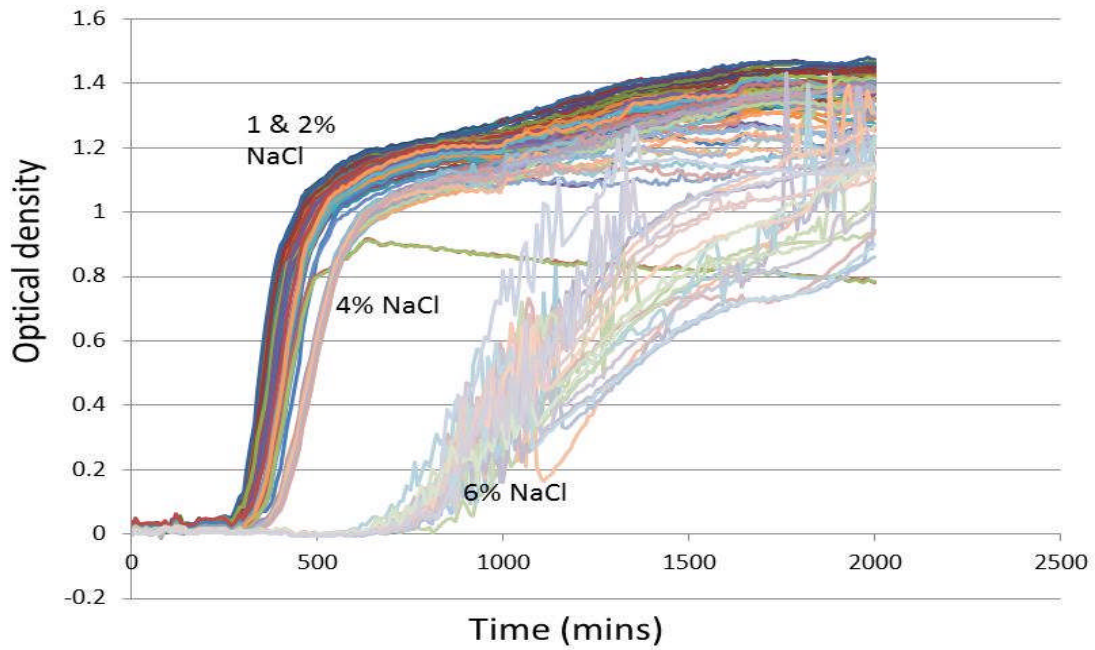
Figure 3-14 Effect of different NaCl concentrations (%) on relative growth of *S.mutans* (8 x 2 replicates) in a TSB broth at 37°C over periods of 2000 mins.
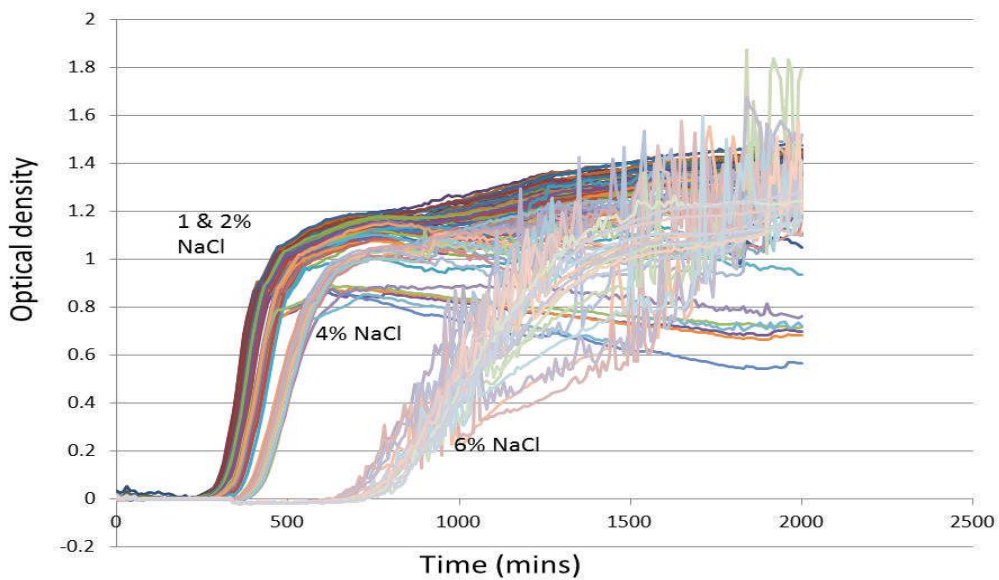
Figure 3-15 Effect of different NaCl concentrations (%) on relative growth of *S.oralis* (8 x 2 replicates) in a TSB broth at 37°C over a period of 2000 mins.

Table 3-7 shows the effect of solute concentration (1-6%) on the TTD for these two species. This shows that they are both very sensitive to >4% NaCl with the lag time prior to growth being almost doubled before growth was initiated.

Table 3-8 The relative Time To Detection (mins) for the effect of pH on *S.mutans* and *S.oralis* based on time required to reach 0.2 optical density at 37°C on TSB medium.

NaCl (%)	*S.mutans*	*S.oralis*
0	322.35	326.75
1	339.39	346.38
2	371.41	374.58
4	422.41	441.86

4 Discussion

In this study we examined the effect of antioxidants and essential oils on growth of two types of bacteria which related to dental caries (*S.mutans; S.oralis*). A total of 9 essential oils and 3 antioxidants were screened in vitro using TSB media at 37°C. Based on zone of inhibition it was found that clove leaf and cinnamon essential oils and BHA and PG antioxidants had the best efficacy against *S.mutans* and *S.oralis* after both 24 and 48 hrs.

Studies by Chaudhari et al. (2012) examined the effect of different ESOs against *S.mutans*. They used 9 commercially available ones in in vitro studies using a blood agar and 37°C. Based on zone of inhibition they found that cinnamon, lemon grass, peppermint, clove and eucalyptus oil had best efficacy against *S.mutans*. They also suggest that they could be effective against oral yeast infections as well. Effects against *S.oralis* were not determined. There appears to be many more studies with *S.mutans* when compared to that on *S.oralis*. The present study suggests that the latter species may have slightly more resistance to the essential oils and antioxidants tested when compared to *S.mutans*.

Yengopal (2009) discussed the potential viability of using essential oils for caries prevention. He suggested that the key to controlling dental caries by *Streptococcus* species, at least one component needs to be effectively controlled, i.e., intervention in relation to the teeth, substrate, or the flora. Thus mouthwashes have been found to effectively reduce microbial counts by simple intervention. However, they predominantly control gram negative microflora, although some, e.g. gingivitis mouthwash, has been shown to control gram positive microorganisms including *S.mutans*.

Takarada et al. (2004) compared the effect of manuka oil, tea tree oil, eucalyptus oil, lavender and romarinus oils against a range of oral microorganisms including *S.mutans*. They found that 0.2% manuka or tea tree

oils were most effective and they also inhibited adhesion by *S.mutans*. Interestingly, it was found to have little effect against cultured human cell lines. Thus they could have potential to be used in formulations of toothpaste.

Propolis is a resinous beehive product which is used by bees as a glue to close the opening of hives. The ethanolic extract of propolis was examined for antimicrobial efficacy against oral microorganisms by Park et al. (1998). They found that this extract inhibited glucosyltransferase activity by *S.mutans* as well as inhibiting growth. They found that the extracts varied from different regions of Brazil and that the components of the extract varied. However, they suggested good potential for controlling such oral microorganisms.

Lee et al. (2011) examined an extract from *Curcuma longa*, a common spice used in oriental foods to control biofilm plaque formation by *S.mutans*. They found that essential oil from this spice inhibited growth of *S.mutans* at 0.5-4 mg/ml. Significant inhibition of biofilm formation on saliva-coated hydroxyapatite beads was also observed using scanning electron microscopy at >0.5 mg/ml concentrations. The key components appeared to be α-turmerone and germacrone (approx. 50%). However, effects against *S.oralis* were not evaluated.

Some studies have concentrated on the inhibition of biofilm formation by bacteria such as *S.mutans*. These studies have often concentrated on inhibition of glycosyltransferases and on the membrane integrity of the bacterium. Thus studies by Koo et al. (2003) examined the effect of apigen and tt-farnesol for these two inhibitory effects respectively. They found that at 1.33 mm apigenin and farnesol the dry weights of biofilms were significantly reduced (by 30-50%) than when treated with control saline solutions. The ability of S. mutans to produce polysaccharides was reduced as well as effects on viability in such biofilms, suggesting potential use in formulations.

Recent studies Hamoud et al. (2012) examined the effect of a complex essential oil distillate (Olbas© Tropfen) against the individual essential oil components (peppermint, eucalyptus, jumiper berry and wintergreen oils) against different microorganisms including *S.oralis* but not *S.mutans*. Against

S.oralis the complex Olbas compound had a MIC of 1.25 mg/ml. However, the individual essential oils had MICs at 10-40 mg/ml. This suggested that interactions between mixtures may affect the final effect in controlling such bacteria. Comparison of different aqueous and organic solvent extracts of teas (oolong, green and black tea) showed that those of oolong and green tea were most effective at inhibiting *S.mutans*. Interestingly, the extracts were more effective than chlorhexidine (Subramaniam et al., 2012). Previously, it was suggested that mixed compounds from green tea combined with indole was very effective against *S.mutans* (Muroi and Kubo, 1993). They suggested that there was a synergistic effect of sesquiterpene hydrocarbons such as cadinene and caryophyllene + indole resulting in a 128 and 256-fold increase in effect on *S.mutans*.

A very recent study by Subramaniam et al. (2012) also examined the effect of pomegranate and aloe vera extract on *S.mutans*. They again used hydroalcoholic extracts of pulp from both *Punica granatum* (pomegranate) and from *Aloe barbadensis* (aloe vera) at 5-100%. There was a significantly better effect of the pomegranate extract on growth of *S.mutans* than the aloe vera extracts. They suggested that this extract is a significant antibacterial agent with potential for control of such oral bacteria.

Polyphenols have been examined for the antimicrobial effects against oral bacteria, especially *S.mutans* (Sendamangalam et al., 2011). They examined natural gallic acid and tannic acid, and salicylic acid and compared this with ascorbic acid, a common antimicrobial compound for comparison. Overall, salicylic acid was the weakest with a MIC of 3.8 mg/l while tannic acid was the best with a MIC of 0.4 mg/ml. They also suggested that the antioxidant properties may contribute to the antimicrobial effects.

In the present study it was found that sodium fluoride at different concentrations (100, 500, 1000ppm) at 37°C had better effect on *S.mutans* than on *S.oralis* populations. The relative efficacy was >75% for *S.mutans*, but only about 25-30% for *S.oralis* at 1000 ppm. This suggests that higher concentrations of sodium fluoride are required. For this reason it was decided to use 2000 ppm

sodium fluoride in subsequent studies on TSB and the artificial saliva medium when testing combinations of the best essential oils or antioxidants + sodium fluoride.

The effect of mixtures of anti-oxidants/essential oils (0.5%, 1% concentration) with sodium fluoride (2000 ppm) on the growth of the two bacteria were interesting. It was found that on TSB the combination treatments completely inhibited growth when compared with the untreated controls regardless of the mixtures used. In contrast, in the artificial saliva medium at 37°C the mixtures were not as effective against both bacteria. Thus, on artificial saliva medium at 1000 ppm some growth of the two bacteria still occurred. The effect of combinations of 0.5% of antioxidants and essential oils + 2000 ppm sodium fluoride were quite effective against both bacteria, significantly reducing the remaining populations after 24 hrs treatment. At 1% the antioxidants/essential oils + sodium fluoride control was much more effective against both *S.mutans* and *S.oralis* after 24 hrs treatment at 37°C in the artificial saliva medium.

The artificial saliva experiments were useful as they showed the potentially more realistic effect of the combined treatments. Saliva is important in as it keeps the ecosystem of the mouth in balance. It contains its own bacterial enzymes that are beneficial in minimising caries causing bacteria. It contains phosphate and calcium ions that help repair teeth. The major organic constituents of saliva are proteins and glycoproteins. Proteins in saliva influence the oral ecosystem. Some may be used as nutrients by bacteria and of course can help wash out caries causing bacteria as far as is possible. Of course, depending on the nutritional balance, especially presence of sugars, this will influence the attachment of caries causing bacteria and the formation of biofilms.

The ecology of both bacteria from the results the growth of the 10 x 2 replicates of pH 7 showed the most rapid growth, followed by pH 6 and 5. At pH 4 growth was the slowest and there was a much longer delay before growth was initiated. The replicates also show a greater variation at this pH . The Time To Detection (TTD) values were calculated and these were found to change as the pH was

made more acidic. This is a good indicator of the effect of pH on the growth of both *S.mutans* and *S.oralis.* and different NaCl concentrations on the growth of *S.mutans* and *S.oralis* on TSB at 37°C. This shows that as the percentage NaCl was increased from 1-2% to 6% the growth was delayed and the rate of growth was slower over the experimental period.

Chen et al. (2012) examined the effect of pH on *S.mutans* growth on denture adhesives in vitro on Polident cream, Protefix cream and Protefix powder. The pH values were measured immediately after preparation and after 1-24 hr intervals. Bacterial growth was observed by measuring absorption at 600 nm every 1 h for 12 h using a spectrophotometer. The tested adhesives generally remained relatively pH-stable over 24 hrs ranging from 5.5 to 7.0. There were no statistically significant differences in *S.mutans* growth rates between the extract-treated and control cultures ($p > 0.05$). However, it has been suggested that S.mutans may be better adapted to lowered pH levels than some other oral bacteria. Thus dominance of *S.mutans* and *S.sobrinus* in resting biofilms at low pH has been suggested because of their ability to survive and remain viable in such a niche. Some have proposed a succession of bacteria in caries that takes into account carbohydrate intake, low pH groups of bacteria and the mutans streptococci (Van Ruyven, 2000). This could mean that a succession of bacteria in a suitable medium can lead to dominance by species such as *S.mutans* which can then lead to caries lesions.

The ability to tolerate a range of ionic salt concentrations may also facilitate the survival and growth of oral caries causing bacteria such as *S.mutans* and *S.oralis*, although less information is available on the latter species (Bowden, 2000). However, water activity of solutions used for oral hygiene can be important in attempts to break up the biofilms and inhibit the activity of these oral caries causing bacteria. The present study suggests that quite high ionic solutions are required to delay or inhibit the growth of these bacteria.

5 Conclusion and future work

- Some essential oils (2 of 9) and antioxidants (2 of 3) have good efficacy to control the growth of *S.mutans* and *S.oralis*

- This study showed that clove leaf and cinnamon oils and BHA and PG were the most effective at 0.5 and 1% concentrations in a TSB medium

- Combinations of 0.5% and 1% of these essential oil/antioxidant treatments with sodium fluoride (2000 ppm) completely inhibited growth of *S.mutans* and *S.oralis* in TSB medium.

- In an artificial saliva medium efficacy was not as effective as on TSB, however the populations of the two oral caries bacteria were significantly reduced by combined treatments

- This suggests that potential exists for using such combinations in formulations of toothpaste or liquid treatments to reduce the growth of such bacteria to improve oral hygiene

- Ecological studies showed the effect of pH (4-7) and NaCl (1-6%) concentration on growth of these two bacteria

- This showed the optimum pH for these two bacteria was 6-7

- The optimum water activity was at 0.99-0.99 (1-2% NaCl concentration).

- Efficacy when biofilms are formed by dental caries bacteria needs to be examined

- Potential for the penetration of biofilms of *Streptococcus* species or mixed inoculate needs to be examined

- Differential efficacy against mixed populations needs to be quantified.

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Appendix I

Figure I.1 shows the effect of different concentrations of initial populations of *S.mutans* on growth profiles in relation to optical density using the Bioscreen system. The range used was 1.4×10^7 to 1.4×10^2 CFUs/ml.
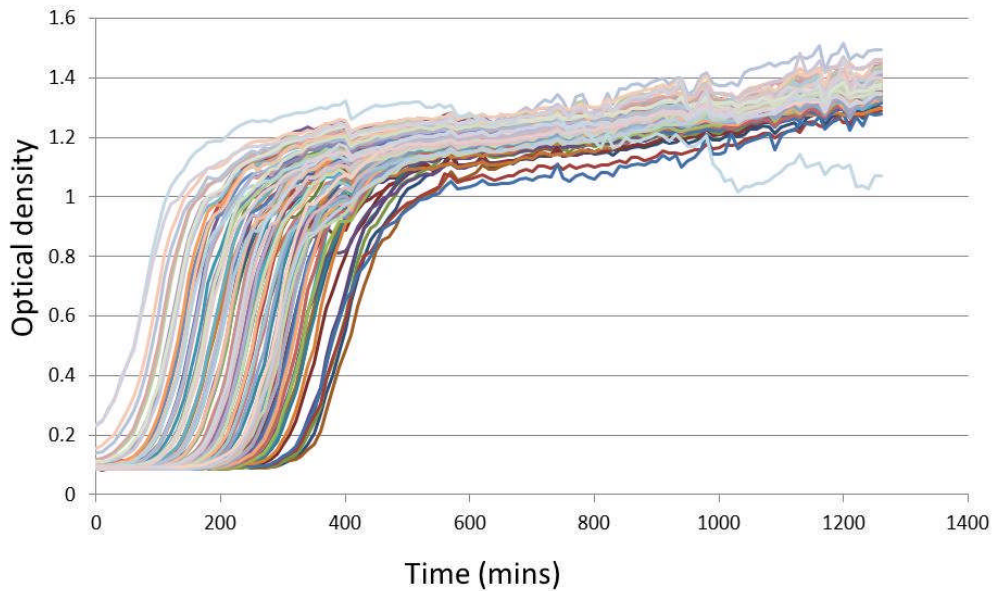

Figure I.2 shows the effect of different concentrations of initial populations of *S.oralis o*n growth profiles in relation to optical density using the BIoScreen over the range of initial concentrations of 5.24×10^7 to 3.3×10^2 CFUs/ml.