In vitro Investigations into the Antimicrobial and Microecological Effects of Selected Anti-plaque Agents

A thesis submitted to the University of Manchester for the degree of

DOCTOR OF PHILOSOPHY

In the

FACULTY OF MEDICAL AND HUMAN SCIENCES

2011

Mohamad M. Mohamad

B.Sc. (Pharmacy)

School of Pharmacy and Pharmaceutical Sciences

CONTENTS

Count: 49,285 words

List of Figures

8

CHAPTER 4

CHAPTER 5

List of Tables

ABSTRACT

The prevalence of oral diseases such as dental caries and periodontitis and the universal need for effective control of oral health has stimulated a great deal of interest in oral hygienic formulations both scientifically and commercially driven. Such formulations are normally deployed as complex formulations commonly containing antimicrobial actives together with excipients, where both classes of ingredients may contribute to the bacteriological effect of the oral hygienic product. However, the mode of action and/or the bacteriological and microecological effects of exposure of microorganisms to oral hygiene products are poorly understood. In this context, this doctoral dissertation represents a series of investigations to contribute to knowledge in the area. The impact of selected oral antimicrobial actives (triclosan, sodium lauryl sulphate, stannous fluoride and zinc lactate) on a key aspect of bacterial cellular membrane function was investigated. This involved measuring major cellular respiratory pathways during exposure to the test agents using two types of tetrazolium dyes possessing different redox potentials as respiration pathway indicators. Spectrophotometric analyses indicated that sub-lethal levels of triclosan and sodium lauryl sulphate act as uncoupling agents, an observation not previously been reported. Sub-lethal concentrations of stannous fluoride and zinc lactate however, blocked cellular respiration with resulting shifts towards glycolytic/fermentative pathways. The contribution of a variety of test agents to the overall antimicrobial effect of a complex formulation (Listerine[®]) was investigated in order to understand the relative efficacy of the actives. This was achieved by testing the essential oils present in the formulation singly and in combination utilising *in vitro* models. The use of the hydroxyapatite disc model (HDM) to grow salivary microcosms to test the efficacy of the ingredients revealed hitherto unreported synergistic activity between the active ingredients thymol and menthol. Proprietary dentifrices (Colgate Total[®] and Crest ProHealth[®]) containing the antimicrobial agents triclosan or stannous fluoride/zinc lactate, respectively, were comparatively evaluated. This was performed by simultaneously establishing salivary microcosms in Sorbarod Biofilm Devices (SBDs). Following the establishment of dynamic steady-states, paired devices were dosed with each of the two proprietary dentifrices. Bacteriological data generated after multiple dosing indicated that both dentifrices were comparably effective in the reduction of all tested bacterial functional groups in the plaque models. However, data generated using HDM models indicated greater reductions in Gram-negative anaerobes after exposure to Colgate Total[®]. The observations presented in this thesis may contribute to the development of oral formulations with optimised antimicrobial efficacies against adventitious pathogens present in the oral cavity and help in reducing the incidence of oral diseases and potentially related systemic interface.

DECLARATION

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

COPYRIGHT STATEMENT

i) The author of this thesis (including any appendices and/or schedules to this thesis) owns certain copyright or related rights in it (the "Copyright") and s/he has given The University of Manchester certain rights to use such Copyright, including for any administrative purposes.

ii) Copies of this thesis, either in full or in extracts and whether in hard or electronic copy, may be made only in accordance with the Copyright, Designs and Patents Act 1988 (as amended) and regulations issued under it or, where appropriate, in accordance with licensing agreements which the university has from time to time. This page must form part of any such copies made.

iii) The ownership of certain Copyright, patents, designs, trademarks and other intellectual property (the "Intellectual Property") and any reproductions of copyright works in the thesis, for example graphs and tables ("Reproductions"), which may be described in this thesis, may not be owned by the author and may be owned by third parties. Such Intellectual Property and Reproductions cannot and must not be made available for use without the prior written permission of the owner(s) of the relevant Intellectual Property and/or Reproductions.

iv) Further information on the conditions under which disclosure, publication and commercialisation of this thesis, the Copyright and any Intellectual Property and/or Reproductions described in it may take place is available in the University IP Policy
(see http://www.campus.manchester.ac.uk/medialibrary/policies/intellectual-property.pdf), in any relevant Thesis restriction declarations deposited in the University Library, The University Library's regulations
(see http://www.manchester.ac.uk/library/aboutus/regulations) and in The University's policy on presentation of Theses.

DEDICATION

To a great man, whom he is

"To my Father"

ACKNOWLEDGEMENTS

There are a number of people without whom this work might not have been produced, and whom I would like to acknowledge below;

My supervisors Dr Andrew McBain and Dr David Allison, it would have been next to impossible to write this thesis without their continuous support and guidance.

My parents whom I am indebted for the rest of my life for their care, love and moral support.

My wife without whom this effort would have worth nothing. Her love, support and patience have taught me so much about sacrifice, discipline and compromise.

My sister and brothers, whom have always been so far but so close within each others hearts.

My twin children Adam and Lana, their happy faces are and will always be the joy of my life.

I would like to express my deep gratitude to the memory of my late supervisor Prof. Peter Gilbert who will always be missed.

CHAPTER 1

General Introduction

1.1 The human microflora

It is clear that the world that we live in is dominated by microorganisms which include unimaginable numbers of bacteria. The human body itself has been estimated to be comprised of over 10^{14} cells, of which only 10% are mammalian and 90% are accounted for by members of the microflora or microbiota (mainly bacteria); (Sanders and Sanders 1984). As a result of the co-evolution between the resident microflora and humans, is the establishment of complex relationships. In such relationships the resident microflora of humans plays a positive role directly and indirectly in the development of normal physiology, in nutrition and in the immune system of the host (Marsh 2000). However, a negative or a passive relationship may develop between opportunistic pathogens of the resident microflora and the habitat with the potential outcome of disease. The composition of the resident microflora varies in different habitats of the body and is considered to be a characteristic of those habitats (e.g.; mouth, skin, gastrointestinal tract, etc.). In spite of the variation in microorganisms between distinct habitats, their taxonomic composition is still relatively consistent between individuals (Marsh 2003). Once colonised and established, the composition of the resident microflora in each distinct habitat remains relatively stable, this stability (also known as microbial homeostasis) is maintained to a considerable degree in spite of the host defence mechanisms and despite regular exposure to stress factors of the environment and microbial immigration (Rasiah *et al.* 2005). Many resident

bacteria grow and survive in a nutrient-sufficient matrix-enclosed ecosystem adherent to host surfaces known as biofilms (Donlan and Costerton 2002). Any substantial changes in the environment (pH changes or immune responses, etc.) might cause a break down of microbial homeostasis of these biofilms and potentially leading to disease (Marsh 1989; Newman 1990). An example of such break down of the microbial homeostasis is the prevalence of dental caries due to dietary carbohydrate intake and the resultant repetitive exposure of teeth enamel to low pH (Marsh *et al.* 1988).

1.2 The oral microbial ecosystem

The existence of various microbial habitats in the oral cavity makes the mouth one of the most diverse ecosystems in the human body. Due to differences in their properties and biological features, each part of the oral cavity supports the growth and the development of a characteristic microbial community. Colonisable tissues that are present in the mouth include soft mucosal surfaces such as the tongue, cheeks, lips and the hard surfaces of teeth. However, certain features of these distinctive habitats do not remain stable during the life-time of an individual; for example the eruption of teeth after first few months of life providing new hard (non-shedding) surfaces for colonisation and the introduction of new sources of nutrients such as gingival crevicular fluid (GCF), during tooth extraction or dental treatment, and the insertion of dentures (Kuramitsu and Ellen 2000). Temporary alterations of the oral ecosystem may be affected by the type and the frequency of the food ingested, by antibiotic treatment, and by saliva and GCF flow (Marsh 1991).

1.3 Hard surfaces (teeth)

Their distinctive physical properties make the teeth one of the few non-shedding sites for microbial colonisation in the human body (Fig. 1.1). The body of the tooth consists of four main parts; *i)* Enamel, which is the outer part of the tooth and also considered to be adapted in order to withstand acids, enzymatic attacks and mastication forces. The primary mineral of the tooth enamel is hydroxyapatite which is mainly composed of crystalline calcium phosphate. *ii)* Dentin, which supports the enamel, although it is less mineralized, it is more physically resilient than enamel due to the elasticity caused by collagen contents. Dentin forms the bulk of the tooth and is responsible for the resistance of the enamel to masticatory forces. *iii)* Cementum covers the root of the tooth. The main role of the cementum is to anchor the tooth to the alveolar bone, via periodontal ligaments. Cementum mainly consists of inorganic material (hydroxyapatite) and organic material (collagen). Finally, *iv)* The pulp, which occupies the central part of the tooth and mainly consists of living connective tissue, nerve fibres, and blood vessels extending to the end of the tooth root. Its main functions are nutritive, sensory and protective. Dental plaque refers to the resident microflora of the teeth and various surfaces in the mouth (as described later in Section 1.10).

1.4 Mucosal surfaces

The soft surfaces in the oral cavity comprise the tongue, palette, cheeks and the lips. Besides their function as taste receptors, both papillae and the taste buds at the dorsum of the tongue provide large surfaces for bacterial colonisation. The physiological structure of the tongue helps in protecting the resident microorganisms from being removed by mechanical force of mastication and

saliva flow. Being continuously bathed with saliva, alongside its unique structure provide anaerobic conditions enabling obligately anaerobic bacteria to grow. The continuous shedding of the stratified squamous epithelium of the palette, cheeks and the lips may affect the colonisation of attached microorganisms (Gibbons and van Houte 1970; Gibbons 1984; Marsh and Martin 1999).
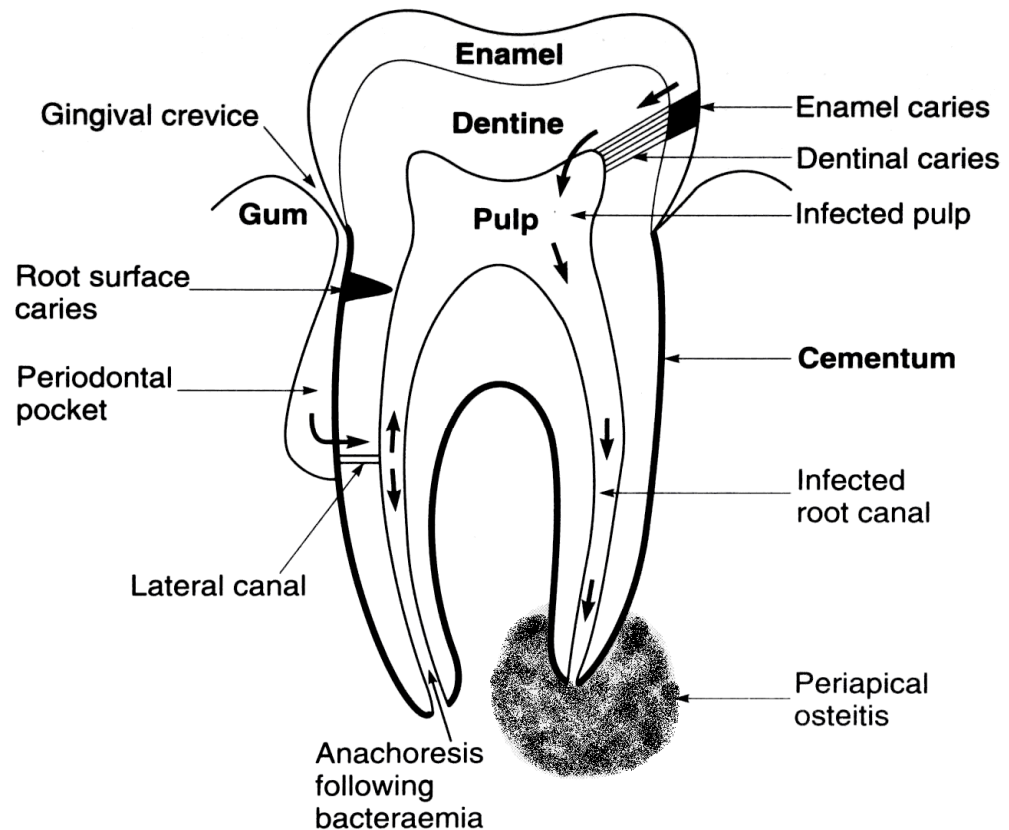
Figure 1.1: Tooth structure in health and disease. Adapted from (Marsh and Martin 1999).

1.5 Saliva

Besides representing a fluid medium for the oral ecosystem, saliva has a major influence as a nutritional source on oral microorganisms (de Jong and Van der Hoeven 1987). Saliva consists of approximately 99% water, with a variety of electrolytes (sodium, calcium, potassium, chloride, magnesium, phosphate, bicarbonate) and proteins (de Almeida *et al.* 2008). Saliva, secreted from the

salivary glands (parotid gland, sub-mandibular gland and sublingual gland) is considered to be part of the total or whole saliva present in the oral cavity, whereas, whole saliva refers to the complex mixture of fluids from the salivary glands, GCF, oral mucosa exudate, the mucous of the nasal cavity and pharynx, planktonic oral microorganisms, dietary residues, shed epithelium and blood cells (Humphrey and Williamson 2001). Normally, mixed saliva has a mean pH of 5.6 to 7.0; averaging 6.7. The pH of the saliva normally increases when its flow is enhanced (Nolte 1982). The flow rate and the concentration of salivary components are subject to circadian rhythms, in which the slowest flow occurs during sleep (Marsh and Martin 1999). The composition of human saliva and the average concentrations of its components are listed in Table 1.1.

The major classes of organic components in saliva are proteins and glycoprotiens. The glycoprotein mucin comprises almost 25% of the total proteins present in saliva. The main types of mucin present in the saliva are MG1 and MG2. Because its is highly glycosylated, mucin type MG1 binds tightly to the enamel of the teeth, forming complexes with other saliva constituents. It is therefore, a major component of the acquired pellicle to which resident microflora attach. Although, MG2 is less glycosylated and more soluble than MG1, it is more easily displaced to form aggregates with the resident microflora (Marsh and Martin 1999; Wilson 2005). Since saliva covers the whole oral cavity with a thin layer of *ca.*100μm in depth, nutrients present in the saliva will be rapidly and continuously transported to the bacteria attached to most oral surfaces.

Table 1.1: The main constituents of stimulated human saliva.

Substance	Concentration (mg/100ml)
Amino acids	3.4-4.8
Ammonia	3.0
Amylase	38.0
Bicarbonate	200.0
Calcium	6.0
cAMP	50.0
Citrate	0.2-2.0
Chloride	100.0
Free carbohydrates	2.0
Glucose	1.0
Glycoprotein	110-300
IgA	19.0
IgG	1.4
IgM	0.2
Lipid	2.0-3.0
Lysozyme	11.0
Peroxidase	0.3
Phosphate	12.0
Potassium	80.0
Sodium	60.0
Thiocyanate	1.0-3.0
Total proteins	140-640
Urea	13.0
Protien	140-640
Uric acid	3.0

Data obtained from (Wilson 2005).

1.6 Gingival Crevicular Fluid (GCF)

Serum components can reach the oral cavity in the form of a transudate fluid flowing via the junctional epethelium of the gingival crevice that is termed gingival crevicular fluid (GCF; Fig. 1.1). The rate of the GCF flow is considered to be relatively slow in healthy individuals (*ca.* 0.3-8µl per tooth per hour), compared to individuals with active periodontal disease. The rate of flow increases markedly in

cases such as gingivitis were it reaches 14µl per tooth per hour and 44µl per tooth per hour in periodontitis cases (Wilson 2005). Gingival crevicular fluid is a rich source of nutrients for microbial inhabitants of the mouth, especially in the subgingival area. The main constituents of the GCF are proteins, and a range of inorganic ions and carbohydrates. Gingival crevicular fluid also contains epithelial and immune cells, which play and important factor in regulating the resident microflora of the gingival crevice in both health and disease (Marsh 2003). Leukocytes are one of the main defence components present in the GCF and GCF is thus a major source of leukocytes in the oral cavity. Major classes of leukocytes are: lymphocytes, monocytes and polymorphonuclear cells (PMNs) which comprise a high percentage. Vitamin K and haemin have also been detected in GCF which are essential for the growth of oral bacteria (Wilson 2005).

In addition to endogenous nutrients provided by the saliva and the GCF, exogenous nutrients present in food play an important role in the metabolism and ecology of the oral cavity. For instance, regular consumption of dietary carbohydrates such as glucose, sucrose, fructose or cooked starch has been reported to be associated with shifts in the balance of plaque and with changes in its metabolic activity (Marsh *et al.* 1988). This shift is mostly associated with increase in the proportion of acidogenic bacteria such as *Streptococcus mutans* and a fall in the proportion of acid-sensitive species (de Stoppelaar *et al.* 1970; Minah *et al.* 1985; Bradshaw and Marsh 1988). Such alterations in the microflora and its metabolism may predispose teeth to dental caries (Boyar and Bowden 1985; Milnes and Bowden 1985).

1.7 Factors affecting microbial homeostasis in the oral cavity

1.7.1 Temperature

The temperature of the mouth is maintained relatively constant, ranging between 35-36°C. Relatively stable temperatures provide suitable environmental conditions for the optimal growth and metabolism of diverse bacterial species. Fedi and Killoy (1992) have reported that the temperature in active periodontal pockets is higher than healthy sites with a mean of 39°C. Higher temperatures can affect gene expression and down-regulate the virulence genes of some periodontal pathogens such as *Porphyromonas gingivalis* (Amano *et al.* 1994; Xie *et al.* 1997). It has also been reported that temperature may vary between different subgingival sites within the same person, thereby affecting the proportion of some bacterial species such as *P. gingivalis, Tannerella forsythensis* and *Campylobacter rectus* (Maiden *et al.* 1998).

1.7.2 Oxygen availability (redox potential)

Although the oral cavity is a highly aerated environment, it is colonised with a majority of either facultatively or obligately anaerobic microorganisms. This is due to the fact that early bacterial colonisers such as streptococci utilise oxygen in their respiratory processes and produce CO_2 (Gottschalk 1986). Anaerobic bacterial species require reduced conditions for their growth/survival. The consumption of O_2 and the production of CO_2, therefore, provides reduced environmental conditions suitable for the growth and metabolism of anaerobic species (later colonisers); (Bradshaw *et al.* 1996). Anaerobic species utilise glycolytic/fermentative pathways with the production of organic acids (pyruvate, lactate) causing highly reduced environmental conditions. Hence, the distribution

of anaerobic bacteria in the oral cavity is highly dependable on redox potential at a specific habitat. The structure of some sites in the oral cavity also has a considerable impact on the redox potential. Various reports indicate that periodontal pockets are more highly reduced than healthy gingival crevices within the same mouth. For example, the posterior surface of the tongue has been found to be more anaerobic than the anterior region (Kuramitsu and Ellen 2000). Therefore, the growth and survival of anaerobic species such *Fusobacterium*, *Porphyromonas* and *Prevotella* on these sites is more likely than less reduced habitats.

1.7.3 pH

The buffering capacity of saliva is responsible for moderating the pH of the oral cavity. This is due to the fact that individuals produce stimulated saliva most of the time which has a neutral pH (Edgar 1976). Normally, stimulated saliva contains proteins, bicarbonates and other constituents, providing the saliva a good buffering activity (Wilson 2005). Neutral pHs are optimal for growth of many types of oral microflora. However, at low pH values, especially in cases of frequent consumption of dietary carbohydrates, saliva may not provide complete protection due to the continuous production of acid by acidogenic species such as streptococci and lactobacilli (Wilson 2005).

1.8 Acquisition of the oral microflora

For a human being, birth represents a borderline between the sterile intra-uterine life and the extra-uterine existence in the local environment, associated with continuous exposure to microorganisms. The colonisation of microorganisms to the infant's oral cavity requires the successive transmission from the environment to the host. The diversity of bacterial composition appears after only few days of life (Socransky and Manganiello 1971). The supply of suitable bacteria by direct and indirect contact with the surroundings of the infant affects the composition of the early colonising microflora. In spite of the colonisation of different species in the oral cavity of the infant, their numbers are not as high compared to adult microflora (Wilson and Henderson 1998). Other species of streptococci, staphylococci, micrococci, lactobacilli, entrococci and neisseria have been detected in lower numbers. However, *Streptococcus salivarius* is generally present at high numbers after one day of birth (Carlsson *et al.* 1970; Socransky and Manganiello 1971; Wilson and Henderson 1998). Streptococci are generally the first colonisers in the oral cavity, whereas *Streptococcus mitis* and *Streptococcus salivarius* are early colonisers within genus streptococci (Kononen *et al.* 2002). Additionally *Actinomyces odontolyticus* is a primary colonizer within the genus *Actinomyces* (Kononen *et al.* 2002; Marsh 2005). It has been also reported that *Veillonella* species and the *Prevotella melaninogenica* group are amongst the first obligate anaerobes. *F. nucleatum,* non-pigmented *Prevotella* species and *Porphyromonas catoniae* have also been reported to be amongst early bacterial communities of the mouth (Marsh 2005). The oral bacterial community becomes more complex and diverse especially after the eruption of teeth. The presence of teeth provides a new habitat of non-shedding surfaces with a new nutrition source

represented by the GCF suitable for biofilm formation and maturation. *Streptococcus mutans* and *Streptococcus sanguis* appear to colonise the mouth after tooth eruption (Kuramitsu and Ellen 2000). Such findings confirm that changes in the environment, such as the emergence of teeth may have a significant impact on the microbial diversity. The continuous direct and indirect contact with family members introduces new species that are normally indigenous in the adults' oral cavity. Gram-negative anaerobic species have been recovered more commonly after teeth eruption. *Prevotella melaninogenica, F. nucleatum,* non-pigmented *Prevotella* species recovery incident was higher in the oral cavity of infants with a mean age of 32 months than previous age 0(Marsh and Martin 1999). An exact time-frame for colonisation of Gram-negative anaerobes and spirochetes has not been firmly established because these species are fastidious and difficult to culture but have been known to be recovered form the oral cavity of children within five years of age (Marsh 2005).

1.9 Functional bacterial groups of the oral cavity

The physiological differences and properties of various habitats in the oral cavity provide a suitable environment for the growth and colonisation of a wide range of bacterial species. Culture-independent approaches such as 16S rRNA analysis have detected *ca.* 500 different microbial species, in addition to 200 species that have been isolated and cultured from the oral cavity (total of *ca.* 700 different taxa) (Paster *et al.* 2001). The proportion of the cultivated species (*ca.* 40%) from the oral cavity is higher than from any other habitat of the human body. It is not uncommon therefore, to culture 20-30 species from different individuals (Paster *et al.* 2001). The prevalence of oral diseases such as dental caries and periodontitis

has stimulated a great interest in studying their aetiology; *i.e.* the bacterial ecosystem and their habitats; to help developing various methods and chemicals for a better oral hygiene control.

1.9.1 Gram-positive cocci

The genus streptococci represent the majority of the resident Gram-positive microflora and are routinely isolated from all the habitats in the oral cavity. The oral streptococci have been differentiated by simple traditional biochemical and physiological tests and more recently by DNA tests. The oral streptococci groups are *i) mutans streptococci* group, members of this group being found in plaque and are mostly associated with dental caries. This group include *S. mutans,* serotypes *c, e, f, Streptococcus sobrinus* serotypes *d, g, Streptococcus cricetus* serotype *a, S. rattus* serotype *b, S. ferus, S. macacae* and *S. downei* serotype *h. ii)* the *S. salivarius* group with members of this group being mostly found on mucosal surfaces and are considered to be related to pathogenesis. This group comprise *S. salivarius* and *S. vestibularis iii) S. anginosus* group, the sub-species presented in this group are found mainly in the gingival crevice and are considered as opportunistic pathogens, and include *S. anginosus*, *S. intermedius* and *S. constellatus. iv)* the *S. mitis* group, members of which are mainly found in dental plaque and include *S. sanguis, S. oralis, S. gordonni, S. parasanguis, S. mitis and S. crista*.

Many species of streptococci have the ability to metabolise sucrose and synthesise extracellular polysaccharides (EPS) such as glucans and fructans. These polymers are essential components of the exploysaccharide-containing matrix of dental plaque as reviewed by (Loesche 1986). The streptococci are

acidogenic and some species such as *S. mutans* are acidophilic, that have the ability to survive at low environmental pH of 4.5 (Loesche 1986; Wilson 2005). The acidophilic and acidogenic characteristics of *S. mutans* strongly implicate this species in the aetiology of dental caries (Loesche 1986). Some species of streptococci have been strongly associated with non-oral infections of the internal organs and are an important cause of purulent diseases. Species of *S. anginosus* have been involved with multiple types of infections in many sites of the body. Pulmonary, liver, central nervous system (CNS) and oral abscesses, as well as infections such as sinusitis, periodontitis, abdominal infections, and skin infections after human wound bites have all been associated with the *S. anginosus* group (Gray 2005).

Despite *Staphylococcus* species have been isolated in low numbers from the oral cavity, they are not considered as part of the normal resident microflora of the mouth (Bagg *et al.* 1995). It has been suggested that *Staphylococcus* species' presence is transient (Smith *et al.* 2001). Although skin and nasal flora are passed continuously, yet these species are unable to colonise or compete against the resident bacteria (Marsh and Martin 1999).

Enterococci, like streptococci species have been isolated in low numbers in the oral cavity and have been strongly associated in many cases of failed endodontic cases (Fouad *et al.* 2002).

Other species of Gram-positive cocci are commonly recovered from carious dentine, root canals and infected pulp chambers. Most of these species are anaerobic and placed in the genus *Peptostreptococcus* such as *P. micros, P. anaerobius* and *P. magnus* (Marsh and Martin 1999).

1.9.2 Gram-negative cocci

The commensal aerobic species *Neisseria* comprise part of the normal microflora of the oral cavity and respiratory tract (Kaplan and Fine 2002). They are isolated from most sites of the oral cavity except the subgingival sites (Aas *et al.* 2005). The most common species is *Neisseria subflava* and they are amongst the primary colonisers of the resident microflora. *N. subflava* produces polysaccharides and is saccharolytic (Marsh and Martin 1999) Although *N. subflava* is not strongly associated with disease, they can however, enter the submucosa and cause opportunistic infections (Kaplan and Fine 2002). In spite of low incidence, several reports have described *N. subflava* as being the causative microorganism of endocarditis, meningitis and septicemia (Lewin and Hughes 1966; Pollack and Mogtader 1984; Amsel and Mouljn 1996; Baraldès *et al.* 2000). Several species of *Veillonella* have been isolated from the oral cavity which are *V. parvula, V. atypica*, and *V. dispar*. These species are anaerobic Gram-negative cocci and are frequently isolated from the tongue, buccal mucosa and dental plaque. The ability of *Veillonella* to utilise metabolites such as pyruvate and lactate as their major carbon source demonstrates the nutritional relationship between *Veillonella* and other oral microflora (Hughes *et al.* 1988), thus contributing to the homeostasis of the ecosystem in different habitats. *Veillonella* species are rarely associated with human infections (Wilson 2005).

1.9.3 Gram-positive rods

Amongst the oral species representing a large proportion of the dental plaque are actinomyces. Seven out of twelve recognised species of actinomyces have been recovered from the human oral cavity and include *A. naeslundii* genospecies 1 and 2, *A. georgiae, A. israelii, A. meyeri, A. gerencseria,* and *A. odontolyticus.*

Actinomyces are Gram-positive, facultatively anaerobic microorganisms which utilise glucose to produce specific organic acids such as succinic, lactic and acetic acids as characteristic end products. In addition, *Actinomyces* are primary colonisers that posses two distinct types of fimbriae (type 1 and 2), which are involved in adherence to the oral cavity tissues and inter-bacterial co-aggregation (Whittaker *et al.* 1996; Amano 2010). *Actinomyces* species are associated with caries (specifically root surface-caries), gingivitis and periodontitis (Loesche and Syed 1978; Brailsford *et al.* 2001).

Despite they only comprise 1% of the total cultivable resident microflora, lactobacilli species are commonly isolated from the oral cavity. They are mostly recovered from human saliva, teeth, the palate, the dorsum of the tongue and the vestibular mucosa (van Houte *et al.* 1972). *L. casei, L. fermentum* and *L. acidophilus* are amongst the most commonly isolated species of lactobacilli from the oral cavity. Since the proportions of these species increase in advanced caries lesion, they were the first organisms to be implicated as specific caries-etiologic agents (van Houte 1994). *Lactobacillus* species are highly acidophilic (van Houte 1980) and acidogenic and have been identified to produce either lactate or acetate (Marsh and Martin 1999).

The genus *Eubacterium* is a poorly-defined group of bacteria. This group comprises of various fastidious, slow growing anaerobes that can be very difficult to isolate and culture. They are mostly abundant in oral infections but rarely found at healthy sites. The oral asaccharolytic *Eubacterium* species are associated with periodontal disease and other oral infections (Spratt *et al.* 1999).

Despite most propionibacteria species are commensal skin microflora, they have been reported to be isolated from the oral cavity including *P. acne* in particular.

Propionibacteria species are obligately anaerobic and can produce propionic acid from glucose fermentation (Marsh and Martin 1999). Several reports have identified *P. propionicus* as being associated with root canal infections (Sundqvist 1992; Sunde *et al.* 2000).

Corynebacterium matruchotii is a Gram-positive short rod microorganism with long filaments and short, thick terminal ends. *Corynebacterium matruchotii* considered to be a bacterium of significance within the oral cavity and comprises the central filament of "corn-cob formations" (formations in which *S. sanguis* bacteria bind to and surround *Corynebacterium matruchotii* to create a corn-cob appearance in dental plaque); (Fig. 1.2). *Corynebacterium matruchotii* can be isolated from dental plaque, although it is not known to be associated with the aetiology of dental diseases (Takazoe *et al.* 1978; Collins 1982).

Rothia species have also been regularly isolated from the oral cavity. Two *Rothia* species are commonly isolated from the dental plaque and the dorsum of the tongue which include *Rothia dentocariosa* and *Rothia mucilaginosa* (Marsh and Martin 1999; Kanzor *et al.* 2002). *Rothia dentocariosa* has been reported to be associated with other infections in the human body such as infective endocarditis (Boudewijns *et al.* 2003).
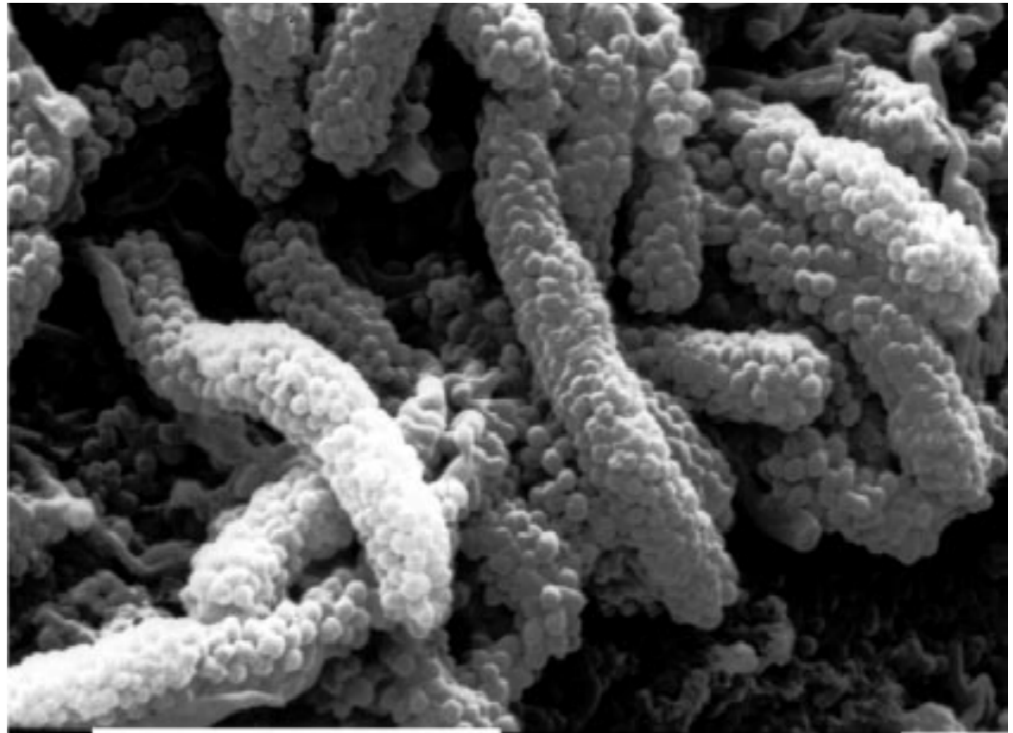
Figure 1.2: "Corn-cob" formations of dental plaque. Image taken from (Sukontapatipark *et al.* 2001).

Bifidobacteria have also been isolated from the human oral cavity in both health and disease. They are Gram-positive, non-motile, non-spore forming, anaerobic bacteria that are generally present in dental plaque (Moore *et al.* 1984; Beerns 1990), gingival crevice (Maeda 1980), and saliva (Sanyal and Russell 1978). *Bifidobacterium dentium* have already been described to be present in dental plaque (Beerens *et al*. 1957) and dental caries (Scardovi and Crociani 1974). More recently two new species of *Bifidobacteria* have been reported to be isolated from dental caries which include *B. denticolens* and *B. inopinatum* (Crociani *et al.* 1996), yet both species have recently been reclassified into two new genera named *Parascardovia denticolens* and *Scardovia inopinata* respectively (Jian and Dong 2002).

1.9.4 Gram-negative rods

1.9.4.1 Facultative anaerobic Gram-negative rods

The majority of this group of microorganisms are represented by *Eikenella, Haemophilus, Actinobacillus* and *Capnocytophaga* species. *Eikenella* species are fastidious facultative anaerobic Gram-negative bacilli species that were first identified by Eiken M. in 1958 (Eiken 1958). *Eikenella corrodens* species are pleomorphic bacilli which sometimes appear in cocci-bacilli shapes and typically create depressions in the agar on which they are growing (Eiken 1958). *E. corrodens* are ccommensal microorganisms of the human mouth and upper respiratory tract and they have been associated in periodontitis (Slots 1977; Socransky 1977) and infective endocarditis (Doref *et al.* 1974).

Aggregatibacter actinomycetemcomitans previously know as *Actinobacillus actinomycetemcomitans* (Nørskov-Lauritsen and Kilian 2006) is another oral commensal Gram-negative rod shaped microorganism. *A. actinomycetemcomitans* is an opportunistic pathogen that possesses certain virulence factors such as leukotoxin that enable it to invade the host tissues. Hence it has been associated in severe cases of localized aggressive periodontitis (Slots and Ting 1999; Haubek *et al.* 2001) and infective endocarditis (Marsh and Martin 1999).

Other species of clinical importance that are present in this category include haemophilli which include *H. parainfluenzae, H. segnis and H. hameolyticus and H. somnus.* Haemophilli are commonly recovered from the saliva, dental plaque and the epithelial surfaces of the oral cavity. Some members of this species have been isolated from infected jaws and have been associated with endocarditis (Darras-Joly *et al.* 1997).

Capnocytophaga species are a group of fusiform, Gram-negative, rod-shaped organisms with gliding motility whose growth is favoured by a capnophilic atmosphere. Normally they are found in human dental plaque and saliva and are also involved in the progression of periodontal disease (Jolivet-Gougeon and Sixou 2007).

1.9.4.2 Obligately anaerobic Gram-negative rods

This category of microorganisms represents a major proportion of the microflora of the oral cavity. Fusobacterium are species of considerable importance and interest in the oral microbiota. These species are characterised by their long filamentous morphology and include a number of oral species that are associated with health and disease such as; *F. sulci, F. periodonticum, F. nucleatum,* and *F. alocis* (Claesson *et al.* 1990; Bradshaw *et al.* 1998). *F. nucleatum* has been identified for its pathological potential in the development of oral disease such as periodontitis due to its number and frequency in periodontal lesions (Dzink *et al.* 1985; Dzink *et al.* 1988; Moore and Moore 1994) and its ability to form aggregates with other pathogens present in the infected site (Kolenbrander and London 1993). *F. nucleatum* can not utilise sugars as the main source of energy (Gharbia and Shah 1988; Robrish *et al.* 1991; Rogers *et al.* 1991), instead it obtains its energy by the fermentation of peptides and amino acids to organic acids such as butyric acid and acetic acid (Robrish and Thompson 1988). However, *F. nucleatum* show no or weak intrinsic proteolytic activity (Brokstad *et al.* 1990), therefore it will profit from its coexistence with other bacterial species such as *Porphyromonas gingivalis* that has the ability to produce proteolytic enzymes and release peptides that are necessary for *F. nucleatum's* growth (Gharbia *et al.* 1989). *F. nucleatum* is

capable of desulfuration of sulfur containing amino acids such as cysteine and methionine, resulting in the release of odorous volatile sulfur compounds (VSC) such as ammonia, hydrogen sulfide, butyric acid, and methyl mercaptan (Pianotti *et al.* 1986; Claesson *et al.* 1990). Methyl mercaptan and hydrogen sulfide account for 90% of the total content (Tonzetich 1977).

P. gingivalis are asaccharolytic, proteolytic species, dependent on nitrogenous substrates for energy such as amino acids and peptides to produces butyrate, acetate and propionate as their main metabolites (Shah and Gharbia 1989). Although sugars such as glucose can be utilized by the organism, they are not converted to metabolic end products but are mainly used for the biosynthesis of intracellular macromolecules (Shah and Williams 1987; Shah and Gharbia 1989; Shah and Gharbia 1993). *P. gingivalis* has an obligate vit. K and iron requirements for its growth (Bramanti and Holt 1990; Wilson 2005). *P. gingivalis* has long been considered as important species of the periodontopathic microflora associated in periodontal disease progression and bone and tissue destruction (Holt *et al.* 1988; Slots and Listgarten 1988). *P. gingivalis* has various virulence factors which include a number of enzymes responsible of the destruction of host tissues such as protease, collagenase, aminopeptidase, gelatinase and nuclease (Holt *et al.* 1999). Along with its destructive enzymes *P. gingivalis* produces a number of odorous cytotoxic substances such as H_2S, butyrate, methylmercaptan and indole (Holt *et al.* 1999)*. P. gingivalis* posses a capsule that protects it from phagocytosis and fimbriae that mediate the bacterium adhesion to its habitat (Holt *et al.* 1999).

Morphologically, both *Prevotella* species and *Porphyromonas* species are similar. However, the main difference is that *Prevotella* are saccharolytic and they have the ability to ferment carbohydrates, producing succinate and acetate. *Prevotella*

species can be categorized into to two either pigmented or non-pigmented main groups. The pigmented group includes *P. loeschii, P. melaninogenica, P. denticola, P. intermedia* and *P. nigrescens* whilst the non-pigmented group include the following species; *P. oulora, P. oralis* and *P. buccalis.* Both *P. nigrescens* and *P. intermedia* have been recognised as being associated with periodontal disease (Socransky and Haffajee 2005; Wilson 2005).

Another microorganism that has been associated with periodontitis is *Tannerella forsythensis* (previously known as *Bacteroides forsythus*). It is a fusiform bacillus microorganism that utilizes amino acids as its major energy source to produce propionic, isovaleric and butyric acid (Wilson 2005). It has been frequently detected with *P. gingivalis* (Klein and Goncalves 2003; Yang 2004) and it can co-aggregate with other species involved in periodontitis such as *P. gingivalis* and *F. nucleatum* which might increase its colonisation in the periodontal pockets (Holt and Ebersole 2005).

Leptotrichia buccalis is another fusiform anaerobic, saccarolytic microorganism that becomes aerotolerant in the presence of CO_2 enriched environment (Wilson 2005). It is usually isolated from dental plaque and plays a role in the etiology of periodontal diseases or in oral-related abscesses (Kondo *et al.* 1978; Reig *et al.* 1985).

Selenomonas noxia, S. fluggei, and *S. artemidis* have been recently discovered in plaque of the gingival crevice (Marsh and Martin 1999). They are saccharolytic, motile microorganisms that have a unique helical shape. *S. noxia* has been strongly associated in cases of periodontitis (Tanner *et al.* 1998).

Several *Campylobacter* species including; *C. rectus, C. concisus, C. sputorum* and *C. showae* have been isolated from the subgingival sites. These species are

assaccharolytic and have a unique spiral shape. They produce succinate as their only metabolic end-product. *C. rectus* has been associated in cases of periodontitis (Macuch and Tanner 2000).

1.9.5 Spirochaetes

Spirochaetes are Gram-negative, anaerobic, motile, helically and highly coiled (spiral-shaped) microorganisms. Oral spirochaetes belong to the genus *Treponema* and include a number of species such as; *T. socranskii, T. denticola, T. vincentii* and *T. microdentium*. They are highly fastidious microorganisms that are very difficult to grow in the laboratory (Dewhirst *et al.* 2001). Spirochaetes can be detected in the subgingival plaque and *T. denticola* in particular has been associated with gingivitis (Lee *et al.* 2005), root canal infections (Rocas and Siqueira 2005) and periodontitis (Choi *et al.* 1993). The association of spirochetes with oral diseases have been accounted for several virulence factors. These virulence factors include; adhesins, trypsin-like proteinase, peptidase and haemosyline (Chan and McLaughlin 1999). The ability *T. denticola* to produce glycine and pyruvate can support the growth of other microorganisms in the microbial habitat (Wilson 2005).

1.10 Dental plaque as a biofilm community

Dental plaque can be defined as a mosaic dense community of microorganisms, embedded in an extracellular matrix of polymers of both host and microbial origin mainly found on various surfaces of the mouth as a biomass (Marsh 2004). Dental plaque has been described in detail on different parameters: *i)* On a clean surface over time. *ii)* In people with different ages, from different countries and diets with precise deficiencies in their host defenses (acquired and innate). *iii)* Following various therapies (Robinson *et al.* 1997).

The composition of dental plaque varies on distinct anatomical surfaces (fissures, approximal, smooth surfaces, gingival crevice and dentures) due to different physical and biological properties of each site (Bowden *et al.* 1975). Dental plaque normally accumulates at stagnant sites that protect it from removal forces applied to the mouth.

For the establishment of a mature plaque community, dental plaque undergoes several phases of development which include: *i) Pellicle formation*, following eruption or cleaning, a conditioning film (the acquired pellicle) forms immediately (Marsh 2004) and influencing the pattern of initial microbial colonization (Al-Hashimi and Levine 1989); *ii) Passive transport of oral bacteria to the habitat*. Reversible adhesion occurs by weak long–range physicochemical interactions between the surfaces of the early bacterial colonisers and the glycoprotein-pellicle of the coated tooth (Busscher and van der Mei 1997), while irreversible adhesion occurs by strong, short-range interactions between specific molecules on the bacterial cell surface (adhesins) and complimentary receptors in the pellicle (Jenkinson and Lamont 1997). With respect to the DLVO theory of colloidal stability, bacterial phenotypic changes are also required to fully explain irreversible

adhesion to oral surfaces. Oral bacteria can participate in multiple interactions at the same time with the host molecules and similar receptors on other bacteria (co-adhesion). In this stage cocci microorganisms are absorbed onto the preconditioned environment, the pioneer species include mostly *Neisseria, streptococci, Actinomymyces* and *Haemophilus* species. These early colonisers grow and multiply forming colonies that become embedded in the biofilm's EPS, creating favourable conditions for the co-adhesion of later coloniser (Marsh 2004); *iii) Co-adhesion of secondary bacterial colonisers to primary colonisers*. A special interbacterial adhesin–receptor interaction is involved in this stage which usually involves lectins. This kind of adhesion usually leads to an increase in the diversity of bacteria in the biofilm and the formation of unusual morphological structures like corn-cob formations (Kolenbrander *et al.* 2000). The process of co-adhesion allows bacteria in dental plaque to engage in a range of antagonistic and synergetic biochemical interactions (Marsh and Bradshaw 1999). Close physical contact of bacteria in dental plaque may enhance the efficiency of metabolic interactions for example; the co-adhesion between anaerobic bacteria and oxygen-consuming species will ensure their survival in an aerobic oral environment (Bradshaw *et al.* 1998), i.e. the consumption of environmental oxygen by early colonisers produces a low redox potential environment creating suitable conditions for the survival and growth of late colonisers. *iv) Multiplication of bacterial colonisers*. Continued growth of cells in dental plaque leads to a three dimensional spatially and functionally organized mixed-culture biofilm. A complex extracellular matrix normally made up of soluble and insoluble glucans, fructans and hetropolymers is formed due to the production of polymers, the formed matrix has the ability to be biologically active and retain nutrients like water, key enzymes

inside the biofilm (Allison 2003). The presence of such matrix plays an important role in the integrity and general resistance of biofilm. Within time the number of early coloniser decreases whilst the proportion of late colonisers (such as *Fusobacterium* and *Veillonella* species) increase. The increase in the proportion of late colonisers causes a further reduction in the redox potential within the growing plaque, which is favoured by more late colonisers ultimately producing a highly complex and diverse bacterial community within an EPS-containing matrix (Fig. 1.3). *v) Detachment and shedding.* Detachment of oral bacteria from surfaces enables many bacteria to re-colonize a new site in the oral cavity. This behaviour can be considered as a response to an environmental cause such as stress, an example of this, is the production of hydrolysing enzymes by some sessile bacteria leading to the hydrolyses of specific adhesins and subsequently detaching bacteria (Marsh 2004).

Studies using confocal laser scanning microscopy (CLSM) have demonstrated an open architecture of dental plaque similar to other biofilms grown in aqueous systems present in the body (Wood *et al.* 2000). This more open architecture is comprised of channels travelling across the plaque biofilm. These channels have important applications for the penetration and distribution of molecules (such as nutrients, end-products) across the ecosystem of the habitat.

Once a mature biofilm is formed, dental plaque remains relatively stable over time which may benefit the host (Marsh 2000). Dental plaque plays an important role in the normal development of the physiology of the host and decreases the chance of infection and colonization by exogenous species, particularly pathogens (colonization resistance), by acting as a barrier (McFarland 2000). Colonization resistance of bacteria in dental plaque includes effective competition for nutrients

and attachment site by resident oral microflora preventing non-plaque species from residing in the targeted habitat. This also involves creating unfavourable growth conditions for exogenous pathogens (McFarland 2000). It is important to study dental plaque on different sites of the tooth due to the distinctive properties and biological features of each habitat which supports the growth and the development of a characteristic microbial community.

1.11 Supragingival plaque

Supragingival plaque can be defined as biofilms that form at any site of the tooth, above the gingival margin level (Fig. 1.1). These biofilms grow in the fissures of the teeth and between adjacent teeth, known as "fissure plaque" and "approximal plaque" respectively. Fissure plaque is mainly comprised of streptococci, especially extracellular polysaccharide-producing species (Marsh and Martin 1999). Usually, a simpler community is found in fissures compared to other tooth surfaces due to more severe environmental conditions such as limited range of nutrients provided by saliva (Marsh *et al.* 1988). Approximal plaque, on the other hand, is mainly dominated by Gram-positive rods, especially *Actinomyces* species although they have high number of *streptococci* species. The redox potential of the approximal sites are generally lower than the fissures of the tooth suggesting the growth of a more diverse plaque community including Gram-negative microorganisms such as *Fusobacterium, Veillonella,* and *Treponema* species (Bowden *et al.* 1975).
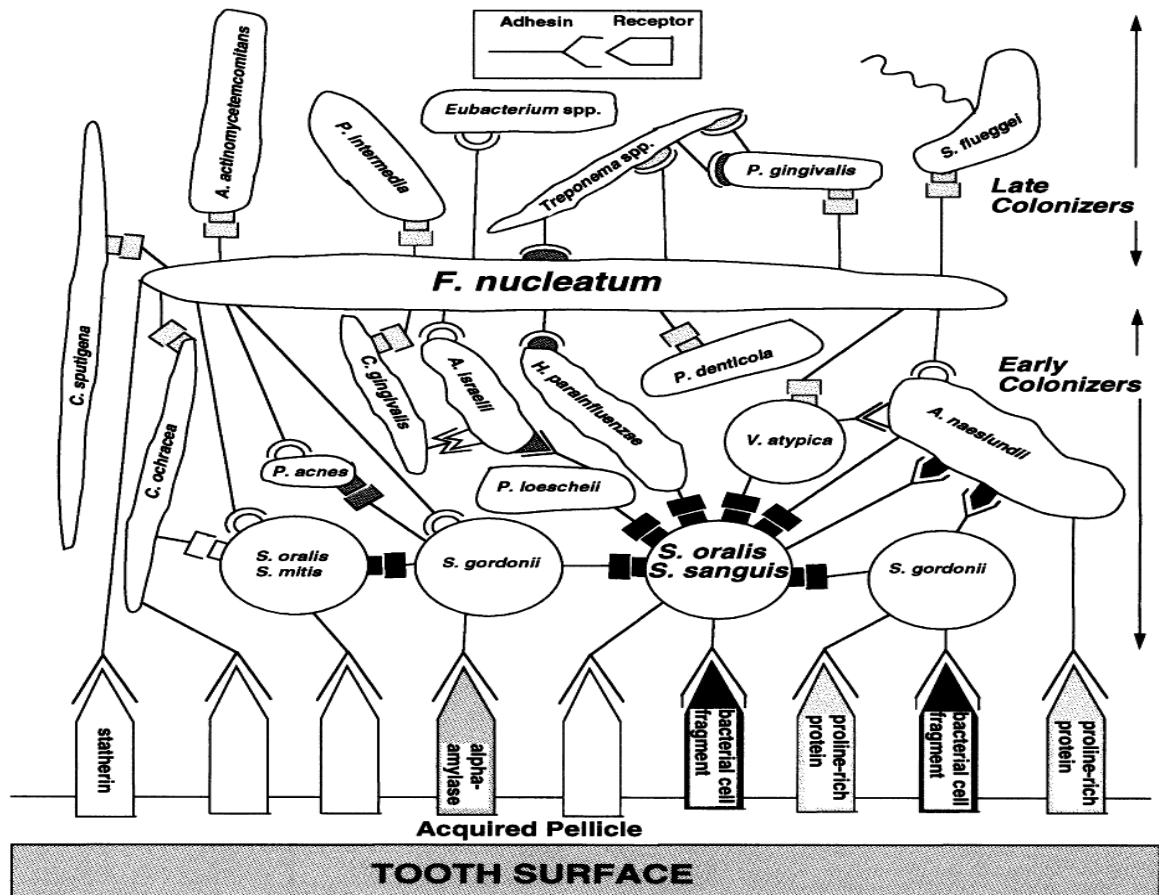
Figure 1.3: Schematic diagram representing the patterns of adhesive interactions between bacteria-host and bacteria-bacteria in dental plaque. Adapted from (Kolenbrander and London 1993).

1.12 Subgingival plaque

This site is a distinct microbial habitat, affected by both the anatomy and the presence of the GCF, which makes it one of the most taxonomically diverse sites in the oral cavity. Many Gram-negative anaerobic bacteria can be found in high levels in this site in contrast to that in fissure and approximal surfaces. Organisms such as spirochetes and anaerobic streptococci can also be isolated from this site. Unlike supragingival sites, most of the microorganisms isolated from this site are assachrolytic and proteolytic and they derive their energy from the degradation of proteins and peptides found in the GCF of the host. *P. melaninogenica, Fusobacterium, Bifidobacterium, Selenomonas*, and *Campylobacter* are some

examples of the microflora that can be isolated from this site (Maeda 1980; Macuch and Tanner 2000). *Streptococcus mitis* group, *streptococcus anginosus* group and Gram-positive rods such as *Actinomyces* species can also be isolated from this site (Loesche 1986; Marsh and Martin 1999; Wilson 2005).

1.13 Role of plaque bacteria in oral diseases

There are two main hypotheses involving the role of dental plaque in the etiology of carries and periodontal diseases.

The first hypothesis is the "specific plaque hypothesis" (Loesche 1992) which suggests that only few bacterial species out of the whole plaque community are responsible for dental caries and other diseases such as periodontitis and halitosis. This hypothesis also proposes targeting a limited number of microorganisms for the prevention and the treatment of a disease (Loesche 1992). The second hypothesis is the "non-specific hypothesis", this hypothesis claimed that dental disease is the outcome of overall activity of the oral microflora, *i.e.* a heterogeneous mixture of microorganisms can play an important role in an oral disease (Theilade 1986).

A later alternative hypothesis has been proposed, which is the "ecological plaque hypothesis". This hypothesis suggested that pathological organisms which are associated with oral diseases can also be found at unaffected areas. This hypothesis also claimed that the dental disease is caused by a shift in the balance of the resident microflora due to changes in the environmental conditions such as repeated conditions of low pH due to the increase of sugar intake leading to a competitive growth of species favouring that environmental condition such as potential caries pathogens (Marsh 1994).

1.13.1 Dental caries

Dental caries is a dynamic and complex biochemical progress that involves a progressive localised destruction of the enamel, dentine and cementum of the tooth. This process is normally caused by bacterial fermentation involved in the production and release of organic acids (pyruvate, lactate, acetate) on the tooth surface, which in turn leads to a pH decline of less than 5.5 and the dissolution and demineralisation of the tooth. The demineralisation process involves the transportation of calcium and phosphate ions away from the tooth to the surrounding environment by the dissolution action of the acids. The demineralisation process can be reversed unless cavities are formed that lead to caries. Dental caries is considered to be one of the more prevalent diseases of humans, particularly amongst populations in industrialised countries. The etiology of dental caries depends on a number of factors, including *i)* plaque microorganisms. *ii)* type of diet consumed by the host, and *iii)* salivary composition and flow rate. Most indigenous plaque bacteria are the microorganisms that are involved in dental caries which include *S. mutans, S. sobrinus* and *lactobacilli* species. All the cariogenic microorganisms that are involved in dental caries have the ability to produce organic acids and survive and flourish in the low pHs that have been created, hence they are acidogenic, acidophilic and or/aciduric. There are four types of caries depending on the affected site of the tooth which include; smooth surface caries, approximal surface caries, fissure caries and root surface caries.

1.13.2 Periodontal disease

Periodontal disease includes many conditions where the supporting tissue of the teeth is affected and may be attacked as a result of a direct action by the microorganisms themselves and/or an indirect action of the inflammatory response which is triggered by the host due to plaque accumulation. The junctional epithelial tissue at the base of the gingival crevice starts to migrate down to the root of the tooth and forming a periodontal pocket and eventually leading to tooth loss.

Gnotobiotic animal studies have provided significant evidence about the role of bacteria as a direct causative factor in periodontal diseases. Those germ-free animals rarely suffer from periodontal disease; however, food can cause a closure of the gingival crevice producing inflammation. This inflammation is more common and severe in the presence of specific pure cultured bacteria isolated from human periodontal pockets used to infect gnotobiotic animal (Marsh and Martin 1999). Bacterial species that are cultured from human periodontal pockets include *Streptococcus, Actinomyces* and Gram-negative species such as *Actinobacillus*, *Prevotella*, *Porphyromonas*, *Capnocytophaga, Eikenella*, *Fusobacterium* and *Selenomonas* species. Studies show that the administration of antibiotics active against pure cultured bacteria of the periodontal disease in the infected animals led to the inhibition of periodontal disease progression (Jordan and Keyes 1972). These findings support the fact that bacteria are implicated in periodontal disease (Marsh and Martin 1999).

1.13.3 Halitosis

Breath malodour or halitosis is mainly caused by volatile odorous substances that are the product of the metabolic and proteolytic activity of the microorganisms accumulated on the tongue surface and the oral cavity. These volatile odorous

substances are present in the air exhaled from the oral cavity or nasal passages of the infected individual (Farrell *et al.* 2006). Although the majority of malodour causes are associated with physiological transient diseases, estimations show that about 10-30 % of the cases may be chronic (Meskin 1996). Other studies show that 85-90 % of all cases have an intra-oral origin (oral malodour), while the rest of the cases have an extra-oral origin which are caused by other infections such as respiratory and gastro-intestinal infections, systemic disease, metabolic disorders or even medication (Tonzetich 1977; Rosenberg 2002).

The predominant VSC that are released by the oral cavity are hydrogen sulphide (H_2S), and methyl mercaptane (CH_3SH), in association with smaller concentrations of dimethyl sulphide $(CH_3)_2S$ and dimethyl disulphide $(CH_3S)_2$. The release of these volatile compounds depends on many factors mainly: *i)* bacterial species population and diversity of the oral cavity, *ii)* substrate availability and *iii)* salivary flow (Sanz *et al.* 2001).

It has been identified that Gram-negative anaerobic bacteria are strongly associated with oral malodour, where high odour individuals normally have higher total Gram-negative bacterial load on the tongue, including species like *Porphyromonas, Prevotella, Fusobacterium* and *Treponema* species (McNamara *et al.* 1972).

Studies by De Boever and Loesche (1995) have shown a positive correlation between the levels of VSC and the amount of biofilm coating the tongue. Later studies showed that the dorsum of the tongue is the main source of VSC in both periodontaly diseased and healthy subjects (Roldan *et al.* 2003).

1.14 Oral antimicrobial agents and the prevention of oral diseases

Oral antimicrobial agents in oral health care products are defined as chemicals that have an effect on plaque sufficient to benefit the oral hygiene and prevent or limit the progression of oral diseases (Lang and Newman 1997). Oral antimicrobials could produce their effect by; *i)* interfering with the adhesion of oral bacteria to surfaces and prevent biofilm formation; *ii)* interfering with the co-aggregation mechanisms; *iii)* affecting bacteria by preventing further growth of colonies, *vi)* removing or causing a disruption in the existing biofilms, *v)* enhancing the local inflammatory and immune responses towards bacteria in biofilms, where some agents enhance the antibacterial mechanism of neutrophils which are considered to be effective against biofilms (Shapira *et al.* 2000).

Guidelines have been proposed by the Council of Scientific Affairs of the American Dental Association (ADA) for the acceptability of antimicrobial agents in oral health products and their control of dental plaque (Sreenivasan and Gaffar 2002). These guidelines state that the use of antimicrobial agents in dentifrices should not result in the growth of pathogenic or opportunistic microorganisms or alter the composition of the oral microflora (ADA 1986). Based on the above guidelines, many studies have been investigating the changes in the proportion of functional groups and resistance of oral microorganisms with long and short term use of both dentifrices and mouthwashes containing antimicrobial agents (Tinanoff and Camosci 1984; Zambon *et al.* 1995; McBain *et al.* 2003; Winkel *et al.* 2003). Therefore, the main objective in the prevention of oral diseases is to maintain equilibrium between the host and the resident microflora, whilst aiming to reduce total biofilm mass and/or the levels of specific groups of opportunistic pathogens (Baehni and Takeuchi 2003). Effective approaches for the prevention include

mechanical removal of plaque (brushing and flossing) with the use of dentifrices and mouthwashes included with antimicrobial agents and also controlling the intake of carbohydrates such as sucrose (Baehni and Takeuchi 2003).

Various oral antimicrobial agents have been introduced to oral care products to enhance their anti-plaque effects. Chlorhexidine, which is an antimicrobial bisbiguianide has been found to have a broad-spectrum antimicrobial effect against a variety of both Gram-positive and Gram-negative bacterial species (Gilbert and Moore 2005). The anti-plaque efficacy of chlorhexidine is owned to its cationic nature that enables it to bind to different surfaces and enabling it to reduce pellicle formation and helping it to act over a long period of time in the oral cavity (McBain *et al.* 2003). Studies by Hase *et al* have demonstrated the efficiency of chlorhexidine in reducing oral bacterial viability (Hase *et al.* 1998), whereas plaque regrowth inhibition and its anti-gingivitis effect was studied by Loe and Schiott (Loe and Schiott 1970).

Essential oils such as menthol, thymol, eucalyptol have also demonstrated both anti-plaque and anti-gingivitis efficacy (Gordon *et al.* 1985; DePaola and Daniel 1989; Daniel *et al.* 1990; Sharma *et al.* 2001; Sharma *et al.* 2004). Studies by Ouhayoun have reported a plaque penetration capability of this mixture of essential oils when incorporated in a proprietary mouthwash formulation (Ouhayoun 2003). Although many studies have reported the anti-plaque efficacy of this formulation, none have investigated the contribution of the active ingredient(s) that maybe responsible for its activity. Therefore, investigating the antimicrobial activity of the active agents in EO-containing (individually and in combination) was one of the objectives and aims of this thesis.

Another antimicrobial molecule that has been tested extensively is the phenolic compound triclosan (Svatun *et al.* 1990; Jenkins *et al.* 1991; Giertsen 2003; Gilbert *et al.* 2003; McBain *et al.* 2010). Triclosan has been incorporated in many oral health products. Besides being a significant anti-plaque and anti-gingivitis agent (Stephen *et al.* 1990; Svatun *et al.* 1990), triclosan has also been reported to posses an anti-inflammatory activity and could broadly suppress multiple inflammatory gene pathways responsible for the pathogenesis of gingivitis and periodontitis (Barros *et al.* 2010).

Metal salts such as stannous fluoride and zinc lactate have also been reported to exert an anti-plaque activity (Skjörland *et al.* 1978; Shah 1982) and have been introduced in many oral hygiene products. Metal salts have a valuable mode of action by inhibiting enzymes responsible for metabolic reactions of carbohydrate and leading to the inhibition of sugar transport and metabolism (Oppermann *et al.* 1980).

Quaternary ammonium compounds such as cetylpyridinium chloride have also been reported to exhibit a significant anti-plaque activity (Holbeche *et al.* 1975; Rawlinson *et al.* 2008). Cetylpyridinium chloride has been introduced in oral care products since the 1940s and have also been proven to have low mammalian toxicity (Arro and Salenstedt 1973).

Further detailed description of the properties, mechanisms of action and antimicrobial efficacies of various investigated antimicrobial agents have taken place within different sections of this thesis.

1.15 Dentifrices & mouthwashes

Dentifrices and mouthwashes are commonly used vehicles for the daily control of oral hygiene as an adjunct to mechanical methods. As previously described in

Section 1.14, a wide range of oral antimicrobial agents have been introduced to dental preparations to provide protection against oral cavity diseases such as dental caries, periodontal disease, gingivitis and halitosis. The antimicrobial agents that have been used in oral preparations belong to various chemically known groups with different mechanisms of action. The most commonly used antimicrobial agents and their mechanism of action are briefly described and listed in Table 1.2.

Table 1.2: The most commonly used antimicrobial agents in dental care formulations

Antimicrobial	Chemical group	Mechanism of action	Use
Chlorhexidine	antiseptic bisbiguanide	Membrane[1] damage	mouthwashes, oral spray, dentifrice, disinfectant
Cetylpyridinium chloride (CPC)	quaternary ammonium compound (QAC)	Membrane[2] damage	mouthwash, dentifrice
Triclosan	phenolic based compound	Membrane[3] damage	dentifrice, hand soup, liquid soup, plastic households
Stannous & zinc slats	metal salts	binding of thiol groups[4]	mouthwash, dentifrice
Hydrogen peroxide (H_2O_2)	antiseptic oxygenating agent	production of active oxygen[5]	mouthwash
Sodium lauryl sulphate	surfactant	initiation of autolysis[6]	foaming agent in dentifrices and mouthwashes
Sodium fluoride (NaF)	fluorinated compound	Cariostatic[7]	mouthwash, dentifrice
Thymol, menthol and eucalyptol	essential oils	biofilm penetration[8]	mouthwash

[1] Davies 1973, [2] Gilbert and Moore 2005, [3] Heath *et al.* 1999, [4] Oppermann *et al.* 1980, [5] Gaffar *et al.* 1997, [6] Gilby and Few 1959; Shafa and Salton 1960; Woldringh and van Iterson 1972; Filip *et al.* 1973, [7] de Leeuw 2004, [8] Ouhayoun 2003.

1.16 *In vitro* modelling of the oral resident microflora

A model is used to represent or mimic real phenomena containing various aspects and elements of the represented system. *In vitro* models have the ability to provide information that explain the process observed in the real system. One objective of a model is to minimise the side/adverse effects that could be produced by experimental antimicrobial agents or antibiotics on human volunteers.

Over the years various *in vitro* models have proven to be useful as substitutes for human volunteers when studying microbial ecosystems of the human body and for providing insight into the effects produced by antimicrobial agents on these communities (Cassels *et al.* 1995; Shu *et al.* 1999; Guggenheim *et al.* 2001; Gilbert *et al.* 2003; McBain *et al.* 2003). There is a range of laboratory experimental oral models used for studying single oral bacteria and/or multi-species biofilm.

These models can be divided into two major groups according to the method of nutrient supply, these are: *i)* Closed system biofilm models: this type of models uses saliva and/or other nutrients incubated with selected species for a specific period of time. An example of a closed system model is the "Zürich" biofilm model that has been developed by Gugggenheim *et al* (Guggenheim *et al.* 2001). In this model, several supragingival plaque species were incubated anaerobically in a saliva-based nutrition medium in which a sterile hydroxyapatite disc was previously introduced in a multi-well tissue culture plate. This type of system is considered to be convenient because it is simple, straightforward to construct, and is relatively easy to manipulate different parameters such as the applied medium, feeding regimens and the added antimicrobial agents (Guggenheim *et al.* 2001). *ii)* Open system biofilm models: this type of system involves a continuous flow of

culture or nutrients and could produce a closer representation to the continuous flow of nutrients and bacterial shedding in the oral cavity. The open systems include various range of models that some of which will be described in the following sections.

1.16.1 Chemostats

The chemostat is considered to be a typical system for producing microbial cultures in steady-state homogenous suspensions (Novick and Szilard 1951; Marsh *et al.* 1983; McDermid *et al.* 1986). Chemostat use for studying biofilms is very limited due to the fact that bacterial cells remain in suspensions rather than biofilm form. However, two-stage chemostat system was developed with colonisable material of Suspended Substratum Biofilm Reactors (SSRs) in a second vessel. The two-stage chemostat was applied for the study of biofilm formation (Keevil *et al.* 1987; Marsh 1995). A limitation of using chemostats for studying planktonic bacterial growth rates is the "wall growth" of biofilms on the surface of the fermentation vessel which serves as a nidus for irregular shedding of bacteria into the cell suspension (McBain 2009). The system is relatively easy to construct and the SSRs can be easily removed and analysed at any time during the experimental run.

1.16.2 The Constant Depth Film Fermenter (CDFF)

The CDFF is an open system biofilm model which allows the formation of large scale of replicate biofilms. In this model, the biofilms are formed on the top of plugs that are installed to a fixed depth in a rotating pan. Any biofilm growing above the recess level of the plugs will be scraped off by a blade keeping the

biofilms in a constant depth (Peters and Wimpenny 1988). The system has several inlets allowing it to be fed by the addition of growth medium whilst other inlets could be used as challenging ports. Different types of substrata, such as polytetrafluoroethylene, hydroxyapatite, tooth enamel and porcelain may be used (Wirthlin *et al.* 2005). The CDFF has been reviewed and widely used by many researchers to study biofilms such as dental plaque (Hope *et al.* 2002; McBain *et al.* 2003; McBain *et al.* 2003; Wood *et al.* 2006; Ledder *et al.* 2009). The model has also been used successfully to investigate the effects of several oral antimicrobial agents against single species biofilm grown on various substrata (Pratten *et al.* 1998; Pratten and Wilson 1999).

1.16.3 The Sorbarod biofilm device (SBD)

The Sorbarod biofilm device (SBD) is another example of the open system biofilm models. The single sorbarod device was first developed by Hodgson and Gilbert (1995) to examine the physiological properties of single species biofilms. The device consists mainly of a cellulose matrix filter plug wrapped inside a paper sleeve. Biofilms are grown within the filters and fed continuously via an inlet port. Viable counts on already established biofilms can take place by sacrificing the filter or by collecting the spent culture fluid (perfusate) after obtaining a steady state without disturbing the biofilm model. An additional challenging port may be added to the system to investigate the efficacy of antibacterial agents against an established biofilms as studies by Parveen *et al.* (Parveen *et al.* 2001). The system has also been used previously to study population dynamics of *Pseudomonas aeruginosa* and *Burkhoderia capacia* (Al-Bakri *et al.* 2004). One of the main advantages of this type of system is that they allow several samples to

be taken during the period of the experiment without disturbing the steady state of the grown biofilm. More detailed description of the SBD system has taken place in Chapter two of this thesis.

1.16.4 The drip flow biofilm reactor

The main principle of the drip flow biofilm reactor is growing biofilms on glass slide surface by dripping growth medium on it. The device consists of four parallel polycarbonate chambers in which standard microscope slides can be situated in (McBain 2009). Modifications can be done to the system by replacing the type of substratum required for the study. Xu *et al.* have previously utilised this model to investigate the role of oxygen availability in determining the local physiological activity of *Pseudomonas aeruginosa* biofilms growing on stainless steel substrata (Xu *et al.* 1998). Hydroxyapatite coated slides in a modified drip flow biofilm reactor have been previously used to evaluate the antibacterial effects of dental hygiene formulations (Ledder *et al.* 2008). Feed-line inconsistencies can however, cause aerial heterogeneity of the biofilm over the substratum and hence, biofilms replication between different chambers may be difficult (McBain 2009).

1.17 Aims

From a superficial perspective the persistence and activities of bacteria in the oral cavity represents a nuisance, requiring daily physical and chemical treatment and, for almost all individuals, requiring visits to the dentist for routine and occasionally unpleasant treatment. However, the carriage of large numbers of microorganisms in the oral cavity, the large bowel or on the skin may be frequently protective

through a process that has been termed colonisation resistance. Importantly however, it has also been associated with potentially serious diseases. A good example of this is periodontal disease which may expose the bloodstream to adventitious pathogens and may also result in systemic complications of chronic inflammation. Thus, the potentially serious health implications of poor oral hygiene have been encapsulated in the relatively new concept of the oral systemic interface (Seymour *et al.* 2007).

The prevalence of dental caries and periodontitis and the universal need for effective routine dental hygiene has stimulated a great deal of interest both scientific and commercially driven.

Ever since the early days of preservation and antisepsis, the use of chemical agents to control and/or remove microorganisms has been a key area of interest, with important potential benefits to humankind. The antibiotic era has seen massive resources dedicated to the discovery and understating of new chemical entities with antibacterial potential. At the same time, the use of less specific antimicrobial agents such as can be broadly classed as antiseptics and disinfectants have been neglected in relative terms. Consequently, there remains much to learn about the mode of action and/or the bacteriological and microecological effects of exposure of microorganisms to this class of compound.

Since toothpastes (dentifrice formulations) and other oral hygienic agents are commonly supplemented with antimicrobial compounds, and these are used frequently, and considering that the incidence of dental caries and periodontitis globally shows no signs of decreasing, the effect of dental hygienic actives and formulations on the oral microbiota requires further investigation. In order to contribute to knowledge in the area, investigations of the oral microbiota should

include those done from the perspective of antimicrobial efficacy, but also in terms of the metabolic effects of lethal and sub-lethal concentrations of actives. Additionally, the mouth is a key area for biofilm formation and dental plaque was arguably the first biofilm that received systematic research attention from microbiologists.

Oral care products differ from most antibiotics in that they are deployed as complex formulations containing ingredients that are, often for regulatory reasons identified as actives or excipients (putatively inactive ingredients) that provide desirable physicochemical properties on the product and which may also contribute significantly to the product efficacy. In general, the effect of excipients on overall effectiveness is poorly understood.

There are several factors associated with dental plaque that contribute towards dental caries and other oral diseases. Principally, the accumulation of bacteria on hard and soft tissues (dental plaque) is a key target of oral hygienic regimes and products, but additionally; *i)* the production of acidic fermentation products contributes to the erosion of enamel and *ii)* the accumulation of periodontal pathogens which contribute to inflammation has been implicated in periodontitis.

This thesis represents a series of investigations aimed to better understand the above issues. In Chapter 3, selected oral health actives were investigated in terms of their impact on bacterial cellular membranes and on the production of potentially harmful fermentation products (such as lactic acid). Since the use of dental hygiene formulations will be associated with both lethal and sub-lethal exposure, this chapter aimed to better understand the potential effect upon cellular respiration/fermentation in different bacterial species in the presence of effective and sub-lethal concentrations of actives and excipients.

In Chapter 4, the contributions of a variety of ingredients (both active and excipeint) to the totality of antibacterial effects of a complex formulation was investigated in order to help better understand the relative contribution of ingredients to the overall effectiveness of the formulation. In order to achieve this, the antibacterial effects of the ingredients were tested singly and in combination to reveal hitherto poorly understood novel additive and synergistic combinations, which may achieve enhanced bacterial inactivation or removal from surfaces.

Chapter 5 utilised a variety of biofilms models to further investigate the effects of formulations containing actives evaluated in Chapter 3 on plaque accumulation, inactivation and taxonomical composition.

When combined, the experimental work presented in this thesis contributes to knowledge in this relatively poorly understood area of pharmaceutical microbiology.

CHAPTER 2

General Experimental Methods

2.1 Chemicals

Unless otherwise specified, all chemicals and reagents used throughout this study were of the purest available grade and were obtained from Sigma-Aldrich Chemical Company (Poole, U.K.) or from British Drug Houses, (BDH) Ltd (London, U.K.). All bacteriological media used during this study were obtained from Oxoid (Oxoid Ltd, Basingstoke, UK) and formulated in accordance with manufacturer's procedures.

2.2 Sterilisation techniques of growth media and Models

Volumes of less than 1L of growth media were heat sterilised by autoclaving at 121°C for 15 min (1 kg.cm^{-2}); (Bridson E.Y 1970). Media volumes larger than 5 L were sterilised at 121°C for a holding period of 45 min. Heat-liable solutions were sterilised by filtration using 0.22 μm nitrocellulose filters (Millipore, Carrigtwohill, Ireland). Biofilm models, fermentation vessels and media delivery systems were sterilized at 121°C for 45 min.

2.3 Microorganisms and culture maintenance

2.3.1 Bacterial species

The bacterial strains tested throughout the study are listed in the table below.

Table 2.1: Bacterial species and strains used throughout the study.

Bacterial specie	Bacterial strain	Reference
Fusobacterium nucleatum	ATCC 10953	(McBain *et al.* 2003)
Prophyromonas gingivalis	W50	(Gilbert *et al.* 2004)
Neisseria subflava	A1078	(McBain *et al.* 2008)
Lactobacillus rhamnosus	AC413	(Ledder *et al.* 2009)
Actinomyces naeslundii	WVU[*]627	(Gilbert *et al.* 2004)
Streptococcus sanguis	NCTC[*]7863	(Gilbert *et al.* 2004)
Streptococcus oralis	NCTC11427	(Ledder *et al.* 2009)
Streptococcus mutans	NCTC10832	(McBain *et al.* 2008)
Veillonella dispar	ATCC[*]177745	(Gilbert *et al.* 2004)
Prevotella oralis	NCTC11459	(McBain *et al.* 2008)
Escherichia coli	C600	Pharmaceutical Microbiology Group, School of Pharmacy and Pharmaceutical Sciences, The University of Manchester

WVU[*]: West Virginia University; NCTC[*]: National Collection of Type Cultures (UK); ATCC[*]: American Type Culture Collection.

Bacterial strains were cryopreserved at -80˚C using cryobead vials (Technical Service Consultants Ltd, Heywood U.K). Bacteria were grown on Wilkins-Chalgren anaerobe agar and broth and incubated anaerobically in a Mark 3 anaerobic work station (Don Whitely Scientific, Shipley, UK) at 37˚C (Gas mix 80% N_2, 10% CO_2 and 10% H_2) except *N. subflava* and *E. coli* which were both grown on nutrient agar and broth and incubated aerobically.

2.3.2 Collection and storage of salivary samples

Saliva samples used were obtained from healthy human donor(s) that had not taken antibiotics for at least 18 months before saliva collection and had no history

of periodontal disease or active caries. All microcosms were inoculated with freshly collected saliva that was collected in sterile universal tubes.

2.4 Bacterial culture media

Oral microcosms were maintained by using a modified artificial saliva medium. Composition was as follows: (gL^{-1} in distilled water): 2.5, porcine type II mucin; 2.0, tryptone; 2.0, bacteriological peptone; 1.0, yeast extract; 0.1, cysteine hydrochloride; 0.35, NaCl; 0.2, KCl; 0.2, $CaCl_2$; 0.001, haemin and 0.0002, Vitamin K1 (Shah and Williams 1976; McBain *et al.* 2002). For the purpose of isolation and enumeration of functional bacterial groups, a variety of selective and non selective agar growth media were used. These media were: trypticase yeast-extract, cysteine, sucrose agar (TYCS); (Van Palenstien and Helderman 1983) for total streptococci, Wilkins-Chalgren (WC) agar was used for total aerobes and total anaerobes, Wilkin-Chalgren agar with Gram-negative (GN) supplement [containing (mg^{-1}); 5.0, haemin; 0.5, menadione; 10, nalidixic acid; 10, vancomycin; and 2.5, sodium succinate] for total Gram-negative anaerobes, Wilkins-Chalgren (WC) agar was used for total aerobes and total anaerobes, and Rogosa agar (RA) for total lactobacilli. All bacteriological media were incubated anaerobically in a Mark 3 anaerobic work station (Don Whitely Scientific, Shipley, UK) at 37˚C (Gas mix 80% N_2, 10% CO_2 and 10% H_2) for up to 7 days except for WC for total aerobes which was incubated aerobically for 3 days at 37˚C in a standard bench-top incubator.

2.5 Enumeration of bacterial cells by viable plate count

The reproducibility of the viable count technique was tested by conducting serial dilutions (1:10) of overnight cultures of *E. coli* C600 in 0.9% sterile saline solution. Aliquots (100 µl) of the dilution series were aseptically spread in quintuplicate on the surface of pre-dried nutrient agar plates using sterile disposable spreaders (Microspec Ltd, Cheshire, UK). Plates were then incubated overnight aerobically at 37°C before counting the number of colony forming units (cfu) for each plate with a yield between 30 and 300 cfu. The number of viable cells (cfu/ml) from the original suspension was calculated by multiplying the mean number of cfu from quintuplicate plates by the dilution factor. The results for each dilution series were subjected to an analysis of variance (ANOVA) test, the results of which are shown in Table 2.2 and Table 2.3.

Table 2.2: Results of viable counts of *E. coli* C600 cell suspensions.

Plate Replicate No.	Dilution Series				
	1	2	3	4	5
1	90	96	90	91	99
2	96	92	99	103	97
3	103	114	82	80	90
4	85	118	88	101	112
5	90	96	101	107	103
Total	464	516	460	482	501
Mean	93	103	92	96	100

Table 2.3: Analysis of variation of viable counts technique showing no statically significant variation.

Source of variation	Degree of freedom	Mean square	Sum of square	F value	P value
Between counts	4	114.560	458.240		
				1.314	0.299
Within counts	20	87.180	1743.600		

2.6 Minimum inhibitory concentrations (MIC) determination by growth rate method

Preparation of inocula for MIC and respiratory activity determinations was done as follows: Single colonies of test microorganisms, previously grown on Wilkins-Chalgren agar were inoculated into sterile Wilkins-Chalgren broth (10ml) contained in 25ml sterile Universal bottles and incubated aerobically at 37˚C with continuous shaking at 100 RPM. According to previous verification, the cultures were incubated at 37˚C for 8 hrs (± 1hr), until they were entering mid exponential phase. Bacterial cultures were diluted 1:100 in sterile Wilkins-Chalgren broth to be used as inocula for antimicrobial susceptibility and respiratory activity determination tests. Different concentrations of water-soluble solutions of antimicrobials were prepared in distilled water and then diluted with double strength Wilkins-Chalgren broth, except for triclosan which was prepared in 100% dimethyl sulphoxide (DMSO) which was used as a co-solvent and then diluted with double strength Wilkins Chalgren broth. Stock antimicrobials concentrations were as follows: Sodium lauryl sulphate ($50mg.ml^{-1}$), stannous fluoride ($10mg.ml^{-1}$), triclosan

(10mg.ml^{-1}) and zinc lactate (50mg.ml^{-1}). All antimicrobial agents in this study were obtained from Colgate-Palmolive (Piscataway, New Jersey, USA) and sterilized by filtration through 0.2 µm pore size (NALGENE$^{™}$, New York, USA) filters and then stored in 25 ml sterile Universals bottles at -70˚C. Sterile Wilkins-Chalgren broth (100 µl) was then added to each well of 96-well microtitre plate (Becton Dickinson, New Jersey, USA). Aliquots (100µl) of each agent were transferred in the first column of wells and mixed. Initial concentrations of each antimicrobial were as follows: Sodium lauryl sulphate (6.25mg.ml^{-1}), stannous fluoride (1.25mg.ml^{-1}), triclosan (0.1mg.ml^{-1}) with maximum of 1% DMSO (Gomez and Maillard 2005) and zinc lactate (6.25mg.ml^{-1}). Doubling dilutions were carried out across the plate till the tenth column in case of triclosan and the eleventh column with other antimicrobials using a multichannel pipette, after each dilution tips were changed, 10 µl of the diluted cultures of *E. coli*, *N. subflava* or *S. oralis* (one species per 96-well plate) were added. The plates were then incubated for 16 hrs at 37˚C in a microtiter plate reader (*PowerWave* Xs, Biotek$^{®}$, Vermont, USA) and growth readings were obtained at 470 nm every 20 mins. Negative and positive controls were also included. Positive controls comprised wells containing only 100µl of diluted over night culture and 100µl of sterile Wilkins-Chalgren broth, while negative controls consisted of sterile Wilkins-Chalgren broth. The eleventh column included 1% DMSO and diluted cultures as a second positive control in all triclosan microdilution assays.

2.7 Minimum inhibitory concentration (MIC) determination by broth dilution endpoint method

The broth dilution endpoint method is commonly used for testing the MICs of antimicrobial agents (Pfaller *et al.* 1994). Inocula preparation for the broth dilution endpoint (BDE) determination of antimicrobial susceptibility was performed as follows: Single colonies of the tested microorganisms, previously grown on Wilkins-Chalgren agar was inoculated into 10 ml of sterile Wilkins-Chalgren broth contained in 25 ml sterile Universal bottles. Anaerobic microorganisms were incubated anaerobically in a Mark 3 anaerobic work station (Don Whitely Scientific, Shipley, UK) at 37˚C (Gas mix 80% N_2, 10% CO_2 and 10% H_2) for 24-72 hrs while aerobic microorganisms were incubated aerobically at 37˚C with continuous shaking at 100 RPM for 8 hrs. Bacterial cultures were diluted 1:100 in double strength sterile Wilkins–Chalgren broth to be used as inocula for antimicrobial susceptibility tests. Stock solutions ($2.0mg.ml^{-1}$) of stannous fluoride and zinc lactate were prepared in distilled water and filter sterilised. Stock solutions of menthol ($0.84mg.ml^{-1}$) and thymol ($1.28mg.ml^{-1}$) were prepared in 43.2% ethanol and filter sterilized. Listerine® (100%) mouthwash was also tested for its antimicrobial activity. Aliquots (100µl) of sterile Wilkins-Chalgren broth was added to the 96-well microtiter plate (Becton Dickinson, New Jersey, USA). Aliquots (100µl) of each agent were transferred to the first column of wells and mixed. Doubling dilutions were carried out across the plate using a multichannel pipette and tips changed after each dilution step. Diluted overnight culture (100µl aliquots; one species per plate) were added to the 96-well microtiter plate to produce the following initial concentrations of each antimicrobial agent: ($1mg.ml^-1$) of stannous fluoride and zinc lactate, menthol ($0.42mg.ml^{-1}$), thymol ($0.64mg.ml^{-1}$) with a maximum concentration of 21.6% ethanol and 50% Listerine® mouthwash. The

plates were then incubated either aerobically or anaerobically at 37°C for 48h. MICs were defined as the endpoint at which no turbidity could be detected compared to controls. MICs determination for each antimicrobial agent was carried out in triplicate in the same 96-well plate. Both negative and positive controls were included. Negative controls included sterile Wilkins-Chalgren broth while positive controls included diluted overnight cultures.

2.8 Respiratory activity determination after long-term (18 hrs) exposure to antimicrobial agents

To assess respiratory activity, solutions of 0.3% of two types of tetrazolium dyes [2,3,5-triphenyl tetrazolium chloride] (TTC) and Iodonitrotetrazolium chloride (INT) Sigma-Aldrich Chemical Company (Poole, U.K.) were added to the microtiter plate after long-term (18 hrs) exposure to different concentrations of different antimicrobial agents (Section 2.6). Tetrazolium salts were prepared as follows: 0.3% of TTC or INT were prepared in 6% D-glucose monohydrate (BDH, Poole, England) and filter sterilized. At the end of the 16 hrs incubation of the microtiter plate, aliquots of 100µl of TTC solution and INT were dispensed in triplicate in different set of rows in the same 96-well plate to produce a final concentration of 0.1% of tetrazolium dye and 2% of D-glucose monohydrate in each well of the microtiter plate. The microtiter plate was then incubated for 1 hr at 37°C in the plate reader and readings were obtained every 2 mins at 550 nm.

2.9 Respiratory activity determination during short-term exposure to antimicrobial agents

Inocula for the determination of respiratory activity during short-term (1 hr) exposure to different concentrations of different antimicrobial agents were prepared as follows: single colonies of tested microorganism previously grown on Wilkins- Chalgren agar were inoculated into 10ml of sterile Wilkins-Chalgren broth contained in25 ml sterile Universal bottles and incubated aerobically at 37°C with continues shaking at 100 RPM. According to previous batch culture studies, the new cultures were incubated at 37°C for 8 hrs (± 1hr), until they were entering mid exponential phase. Antimicrobial agent preparation and initial concentrations were as described in Section 2.6. Aliquots (100μl) of mid exponential phase cultures of *E. coli*, *N. subflava* or *S. oralis* (one species per 96-well plate) were added to each microtiter plate. Freshly prepared, filter sterilized tetrazolium solutions included 0.3% of either INT or TTC and 6% of one of the following substrates: sodium acetate, pyruvic acid (α-ketopropinic acid) and D-glucose monohydrate (BDH, Poole, England). Aliquots (100μl) of TTC solution and INT substrate solutions (one substrate per 96-well plate) were added in triplicate in different set of rows in the same 96-well plate to produce a final concentration of 0.1% of tetrazolium dye and 2% of substrate in the tested wells. The microtiter plate was then incubated for 1 hr at 37°C in the plate reader and readings were obtained every 2 mins at 550 nm.

2.10 Fractional inhibitory concentration method (Checkerboard microdilution method)

The FIC index has been previously used to evaluate combinatorial activities of multiple antimicrobial agents (Hall *et al.* 1983). Inocula preparation and incubation time were previously mentioned in Section 2.7. The fractional inhibitory concentration method was assessed for the two metal salts and all essential oil compounds in separate experiments. Stock solutions (4.0mg.ml⁻1) of stannous fluoride and zinc lactate were prepared in distilled water and filter sterilized. Stock solutions of menthol (1.68mg.ml^{-1}) and thymol (2.56mg.ml^{-1}) were prepared in 43.2% ethanol (twice the percentage present in Listerine®) and also sterilized by filtration. Doubling dilutions were carried out horizontally across the plate for the first antimicrobial agent and vertically for the second agent using a multichannel pipette (stannous fluoride vs. zinc lactate and thymol vs. menthol). Aliquots (100µl) of the diluted overnight culture (one specie per plate) were added to the 96-well microtiter plate to produce the following initial concentrations of each antimicrobial: (1.0mg.ml⁻1) of stannous fluoride and zinc lactate, menthol (0.42mg.ml^{-1}), thymol (0.64mg.ml^{-1}) with a maximum concentration of 21.6% ethanol (equal to the percentage present in Listerine®). The plates were then incubated aerobically and anaerobically at 37˚C for 48h and each MIC determination was carried out in triplicate for each tested bacteria. Negative and positive controls were also included.

2.11 Minimum bactericidal concentration determination (MBC)

The method of Taylor *et al.* was used as the basis for MBC determinations (Taylor *et al.* 1983). These were determined by taking aliquots (10µl) from each well without turbidity of the MIC plate including the end point which are then spot-plated

on Wilkins-Chalgren agar and incubated either aerobically or anaerobically at 37˚C, in triplicate. The MBC was determined as the lowest concentration of tested antimicrobial or tested combination of antimicrobials that produced no growth on the Wilkins-Chalgren agar plate after 3-7 days of incubation.

2.12 Hydroxyapatite disc model (HDM)

The hydroxyapatite disc model has been utilised in many studies to grow bacterial biofilms (Ledder *et al.* 2006; Ledder *et al.* 2009; McBain *et al.* 2010). Sterile hydroxyapatite (HA) discs having a diameter of 9.6mm (Clarkson chromatography, Pennsylvania, USA) were placed aseptically in a sterile 24-well tissue culture plate (Becton Dickinson labware, New Jersey, USA). For validation studies, each disc (n=12) was submerged in one millilitre of 1:100 diluted overnight culture or fresh human saliva and incubated anaerobically in a Mark 3 anaerobic work station (Don Whitely Scientific, Shipley, UK) at 37˚C (Gas mix 80% N_2, 10% CO_2 and 10% H_2) for 48h. The tissue culture plates were removed from the anaerobic station and each HA disc was removed and aseptically submerged in a test formulation or mouthwash for 1 min. before rinsing with sterile PBS (1 min). Each disc was then vortexed for 20 seconds in 9ml pre-reduced half strength sterile thioglycolate broth contained in 25ml Universal bottles with sterile glass beads before serially diluting in half strength broth and plating out onto Wilkins-Chalgren agar, Wilkins-Chalgren agar with Gram-negative supplements, TYCS agar and Rogosa agar plates. All bacteriological media were incubated anaerobically in a Mark 3 anaerobic work station at 37˚ for up to 7 days except for Wilkins-Chalgren for total aerobes counts which were incubated aerobically for 3 days at 37˚C in a standard bench-top incubator. Positive control discs and discs used for validation studies were not

submerged in the mouthwashes. Each validation assay and antibacterial test was carried out in triplicate using a different HA disc in each trial, and each trial was also carried out in triplicate.

2.13 Glass slide biofilm model (GSM)

Glass slides (10 mm^2 x 1mm) were used as an alternative test surface to test for bacterial biomass reproducibility on glass slides pre- and post-antimicrobial treatment. Sterile glass slides (n=12) were placed aseptically in a sterile 24-well tissue culture plates and submerged in one millilitre of 1:100 diluted overnight axenic cultures or one millilitre of fresh human saliva. Culture wells containing fresh saliva or anaerobe bacterial cultures were then incubated anaerobically in a Mark 3 anaerobic work station (Don Whitely Scientific, Shipley, UK) at 37˚C (Gas mix 80% N_2, 10% CO_2 and 10% H_2) for 48 hrs, whilst culture plates with aerobic bacterial species were incubated aerobically for 48 hrs at 37˚C in a standard bench-top incubator. The 24-well plates were then removed from the incubators and each slide was aseptically submerged in sterile PBS (1min). Each slide was then vortexed for 20 seconds in 9ml pre-reduced half strength sterile thioglycolate broth contained in a 25ml universal bottle with sterile glass beads before serially diluting in half strength broth and plating out on a range of different selective agar as described previously (Section 2.4). Each validation assay was carried out in triplicate using a different glass slide in each trial, in triplicate.

2.14 Crystal violet assay (biomass susceptibility assay)

The biomass susceptibility assay has been previously described (Moskowitz *et al.* 2004). A modified assay was performed as follows; Aliquots (150µl) of fresh human saliva were added to the wells of the first 7 columns of two flat–bottom 96-well (12 columns x 8 rows) microtiter plates (Nalgene Nunc International, Rochester, N.Y), whilst 150µl of heat sterilised saliva was added to the wells of the eighth column. Bacterial biofilms were formed by immersing peg lids (Nunc TSP system) into the saliva-incubated plates and incubating anaerobically in a Mark 3 anaerobic work station (Don Whitely Scientific, Shipley, UK) at 37˚C (Gas mix 80% N_2, 10% CO_2 and 10% H_2) for 48h for the first plate, whilst the second plate was incubated aerobically at 37˚C in a standard bench-top incubator for the same period of time. Each peg lid was then placed in a new flat-bottom 96-well plate containing 200µl per well PBS for rinsing for a period of 1 min. to remove loosely adhered or unattached biomass. Each peg lid was then exposed to challenging wells for one min. containing the following concentrations of chemicals and antimicrobial agents; 100% PBS solution (1st and 8th column), 100% Listerine[®] mouthwash (2nd column), ethanol 21.6% (3rd column), 0.042% menthol (4th column), 0.092% eucalyptol (5th column), 0.064% thymol (6th column), and 0.060% methyl salicylate (7th column). Each peg lid was then placed in flat bottom 96-well plate containing 200µl per well of 0.1% crystal violet solution for a period of 15 minutes before rinsing (1min) in a new flat-bottom 96-well plate containing 200µl per well PBS (3 times) and drying (2 hours). To solubilise the absorbed crystal violet, each stained peg lid was placed in a new flat-bottom 96-well plate containing 200µl of 95% ethanol for a priod of 15minutes. The absorbance of each

solubilised crystal violet plate was then read at 590nm in a microtiter plate reader (*PowerWave* Xs, Biotek[®], Vermont, USA).

2.15 Microscopic evaluation of bacterial biomass

Biofilms formed on glass slides were prepared as previously described (2.11). To evaluate the antibacterial efficacy of a proprietary mouthwash on salivary-derived microcosms, each glass slide (n=4) was aseptically removed from the culture plate and submerged in the designated mouthwash for one min before submerging for a further min. in sterile PBS (0.1 M, pH 7) for the removal of excessive antimicrobial agent. Each glass slide was then stained with a LIVE/DEAD bacterial-viability stain (BacLight; Molecular Probes, Leiden, The Netherlands) in accordance to manufacturer's instructions. Once stained, slides were left to dry before washing with 100µl of PBS for the removal of excessive stain. Each glass slide was mounted on an additional glass slide, nail varnish being used as an adhesive on the corners of each slide to prevent the treated glass slide from being displaced during microscopic examination. The glass slides were mounted with a cover slip and immersion oil before examination was carried out using the 100x oil-immersion lens of an epifluorescence microscope (Axioskop 2, Zeiss, Hertfordshire, U.K). Cells were scored as live (green) or dead (red) in ten random fields. Positive controls were also included and contained glass slides submerged in sterile PBS alone and stained according to manufacturer's instructions.

2.16 Sorbarod Biofilm Device (SBD)

The Sorbarod Biofilm Device has been previously adopted for the growth of bacterial biofilms (Hodgson and Gilbert 1995; Al-Bakri *et al.* 2004). Salivary microorganisms were grown within sorbarod devices which were first described by Hodgson (Hodgson and Gilbert 1995). Single sorbarods (Ilacon Ltd, Kent, UK) were inserted in a clear PVC tubing of lengths (40 mm in length, 10 mm in diameter) and sterilised by autoclaving. Sorbarod filters consist of cylindrical paper sleeves encasing a matrix of cellulose fibres. Each assembly was inoculated dropwise with one millilitre of fresh human saliva using a sterile syringe. A two ml sterile syringe plunger (Becton Dickinson, Ireland) was withdrawn leaving the rubber piston within the syringe lumen. The syringe lumen containing the rubber plunger was introduced into the PVC tubing in which the Sorbarod filter was introduced, and a sterile, disposable needle (0.8 x 40 mm) was inserted through the rubber piston. The Sorbarod assembly was then inserted into a plastic funnel (Figs. 2.1, 2.2) to maintain an aseptic environment for the filter and perfusate. Another sterile needle was inserted in the rubber seal to serve as a challenge port. Four Sorbarod units were assembled and set upright and placed within a 37°C incubator. Media inlet tubing was connected to the needles and sterile artificial saliva was delivered to each unit at 0.07 ml/min via a previously calibrated peristaltic pump, (Watson Marlow Model 505S Watson Marlow Ltd, Cornwall, UK). The developed bacterial communities were characterised over a period of one week after inoculation (9 days in total). After duplicate salivary communities had been stabilised and characterised, different antimicrobial suspensions were added to the bacterial communities in the units through the challenge port. The antimicrobials used to challenge the bacterial fermenters were; suspensions of 5%

w/v of Colgate Total® toothpaste and 5% w/v of Crest ProHealth® toothpaste each of which was added to duplicate units separately. Each dentifrice suspension was introduced to the developed biofilms in a rate to reach a maximum of 3g twice a day which resembles roughly the actual amount of toothpaste that is introduce to the oral cavity in a daily regimen.

2.17 Sampling of the Single Sorbarod device

Perfusates from each assembly unit was obtained by detaching the unit from its collection container and placing a sterile universal tube below the outlet, to collect 2ml of the perfusate without affecting the flow rate. For enumeration, perfusate of each model unit was serially diluted using half-strength thioglycolate solution. Aliquots (100µl) of appropriate dilutions were plated in triplicate onto a variety of selective and non selective agar (Section 2.4). Plates were then incubated anaerobically in a Mark 3 anaerobic work station (Don Whitely Scientific, Shipley, UK) at 37˚C (Gas mix 80% N_2, 10% CO_2 and 10% H_2) for up to 7 days except for WC for total aerobes counts which were incubated aerobically for 3 days at 37˚C in a standard bench-top incubator.
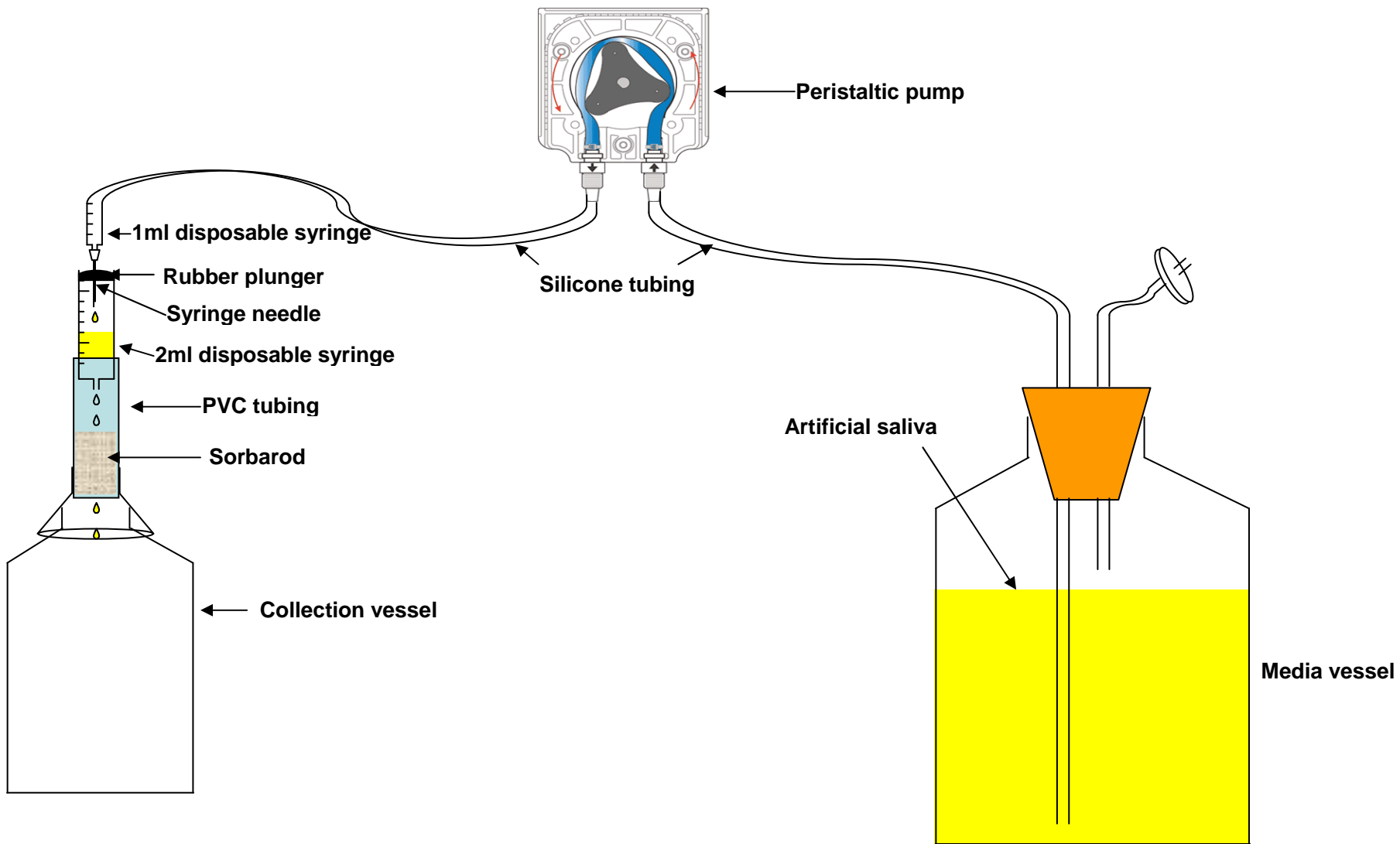
Figure 2.1: Schematic diagram of the Sorbarod Biofilm Model, showing its various parts and the medium feed section (media delivery system).
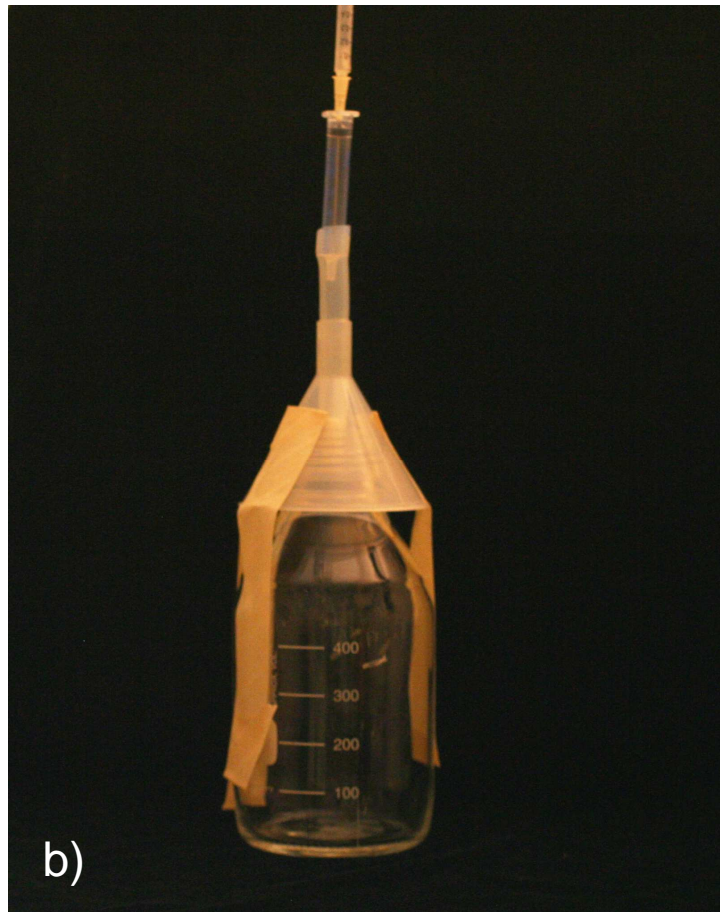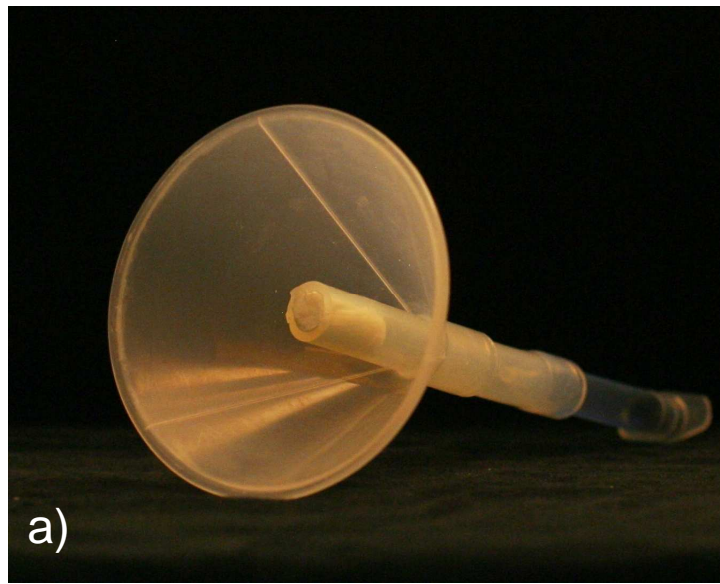
Figure 2.2: Sorbarod plug contained in PVC tubing inserted in a plastic funnel (a). The assembled unit was placed upside down onto a collection vessel and attached with autoclave strips.

CHAPTER 3

Effects of sub-lethal concentrations of various oral anti-plaque agents on bacterial growth and respiratory activity in *Escherichia coli*, *Neisseria subflava* and *Streptococcus sanguis*

3.1 Introduction

The control of biofilm accumulation in the oral cavity by physical removal has been a cornerstone for the maintenance of oral hygiene. Despite its important role in the prevention of gingivitis, periodontitis and dental caries, mechanical removal of plaque is not efficiently practiced by many individuals (Morris and Steele 1998). According to a survey conducted in the United Kingdom, 72% of the examined subjects showed visible plaque and calculus (calcified plaque) on at least one tooth (Morris and Steele 1998). Another limitation associated with the removal of dental plaque by mechanical means alone is that these methods concentrate mainly on the hard surfaces and to considerably lesser extent on the soft tissues in the oral cavity. Although teeth provide an excellent surface for the growth of oral microorganisms, they represent a small proportion of the total surface area of the oral cavity. Previous studies have shown that bacteria involved in oral disease also accumulate on the soft tissues of the mouth and can serve as nidus for colonisation on the hard tissues (Socransky and Haffajee 2005). Poor plaque removal and the incidence of periodontal disease would suggest that mechanical plaque control alone is frequently insufficient. Therefore, the adjunct use of oral care formulations such as dentifrices containing anti-plaque agents is considered to be beneficial for the control of biofilms on different surfaces in the mouth and may contribute to the prevention of oral diseases. Dentifrices used for routine

patient-directed control of oral hygiene include various active ingredients such as metal salts (for example, stannous fluoride and zinc lactate), phenolic compounds (such as triclosan) and surfactants (such as sodium lauryl sulphate). In spite of the long and extensive use of these antimicrobial agents and the clinical proof of their efficacy, there remains a lack of understanding of their mechanisms of action at both effective and sub-lethal concentrations (sub-MIC levels). Studies on sub-MIC levels of such compounds are of relevance because antimicrobial agents delivered from dental formulations may persist at effective levels for only a relatively short period of time in the oral cavity before they are either washed away or swallowed (Marsh 1994) and thus may commonly be present at low concentrations.

3.1.1 Triclosan

Triclosan (2,4,4´–trichloro-2´–hydrodiphenyl ether) is a non-ionic, off-white, odourless and tasteless powder with the molecular formula $C_{12}H_{17}Cl_3O_2$ (Fig. 3.1); (Chemical Abstracts No. 3380-34-5). It is a broad-spectrum antimicrobial agent and it is effective against many Gram-positive (including some mycobacteria) and Gram-negative bacteria (Heath *et al.* 1999). It has been used in skin care products for more than 30 years, and was first introduced in toothpastes in 1985 (Jones *et al.* 2000).
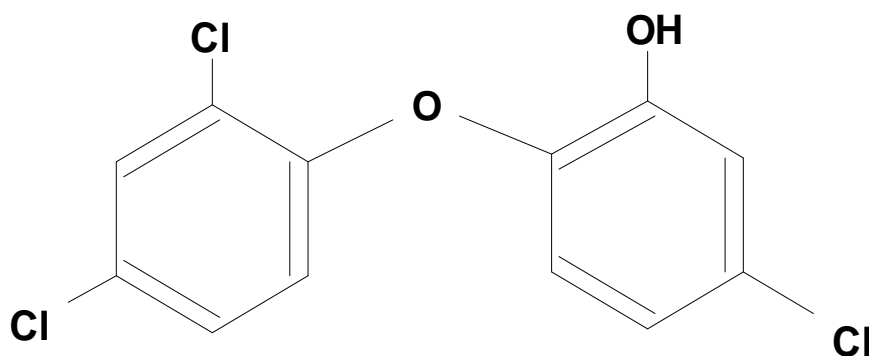
Figure 3.1: The Chemical Structure of Triclosan

McMurry *et al.* (1998) have demonstrated that triclosan binds to the bacterial enoyl-acyl carrier protein reductase enzyme (*fabI*) which is a key enzyme in bacterial fatty acid synthesis (McMurry *et al.* 1998). This binding increases the enzyme's affinity for nicotinamide adenine dinucleotide (NAD^+), resulting in the formation of a stable ternary complex of ENR-NAD^+-triclosan. This complex is unable to participate in the elongation steps in fatty acids biosynthesis (McMurry *et al.* 1998). Such inhibition of membrane biogenesis by the blocking of fatty acid synthesis is associated with the bacteriostatic effect of triclosan whilst its bactericidal effect is attributed to the leakage of intracellular materials (Suller and Russell 2000; Gomez and Maillard 2005).

3.1.2 Stannous Fluoride

Fluorides were introduced to some domestic water supplies in the U.K in the 1940s to improve oral hygiene within the population (Ogaard *et al.* 1994). Stannous fluoride has been used in oral hygiene formulations to prevent caries since 1950s. Stannous fluoride is a broad-spectrum antimicrobial agent exerting its activity against both Gram-positive and Gram-negative bacteria and significantly, has potent activity against the dental pathogen *Streptococcus mutans* (Weber *et al.* 1995). The antimicrobial effect of stannous fluoride is believed to be principally due to the stannous ion rather than the fluoride ion (Ogaard *et al.* 1994). The effect of stannous fluoride on oral bacteria has been attributed to *i)* tin, the polyvalent cation, which may inhibit plaque formation by interacting with negatively charged components in plaque essential for adhesion/cohesion (Skjörland *et al.* 1978); *ii)* the oxidation of enzymes containing thiol groups, causing inhibition of carbohydrates metabolism (Oppermann *et al.* 1980) and reducing DNA

81

transcription (Tinanoff and Camosci 1984; Tinanoff and Zameck 1987). Additionally, the fluoride ion in the compound may react with the hydroxyapatite of the enamel by three mechanisms and thus augmenting the cariostatic effect exhibited by stannous fluoride. These mechanisms are: *i)* ion exchange of F^- for OH^-, *ii)* the formation of fluorapatite crystals from super saturated solutions which is a less soluble compound than hydroxyapatite, and *iii)* the formation of calcium fluoride and its deposition on the enamel (White and Nancollas 1990). The first two reactions may occur during long-term exposure to low levels of fluoride solutions such as in supplemented water supplies, while calcium fluoride formation is the major reaction product of short-term exposure of enamel to concentrated fluoride products (Ogaard *et al.* 1994). When pH levels drop on plaque surfaces, a pellicle protein coat coating the calcium fluoride is lost (Chander *et al.* 1982) leading to the release of fluoride ions that may be adsorbed to the enamel surface and an increase in the rate of remineralisation of the lost minerals (Ogaard *et al.* 1994).

3.1.3 Zinc Lactate

Zinc salts have been recognized for their anti-plaque properties in dentifrices since the 1940s (Hanke 1940; Shah 1982). Zinc is one of the minor elements present in human dental enamel and it is a micronutrient, essential for the growth of microorganisms present in the oral cavity at trace levels. Elevated concentrations of zinc may have inhibitory effects on bacterial growth (Gallagher and Cutress 1977; Babich and Stotzky 1978). Zinc is therefore an effective bacteriostatic antimicrobial agent against caries-associated *Streptococcus mutans* and various oral bacteria that are associated with gingivitis such as *Fusobacterium nucleatum* and *Prevotella intermedia* (Marquis *et al.* 2005). The bacteriostatic activity of zinc

is associated with its affinity for thiol groups present in catabolic enzymes (Oppermann *et al.* 1980). *In vitro* studies showed that the combination of zinc salts with other agents such as triclosan used in dentifrices had a greater inhibitory effect, particularly against Gram-negative species, than that of each agent dosed singly (Cummins *et al.* 1993). Studies have also shown that zinc has an anti-halitosis effect (Yaegaki and Suetaka 1989; Waler 1997). Waler *et al* reported that the mechanism of action zinc involved in the inhibition of volatile sulphur compounds (VSC) production responsible for malodour (halitosis) was due to its ability to form stable mercaptides with the substrate of the VSC in the oral cavity rather than its affinity to oxidize enzymatic thiol groups (Waler 1997; Young *et al.* 2001).

3.1.4 Sodium Lauryl Sulphate

Sodium lauryl sulphate (SLS) is an anionic detergent that has been used singly or in combination with other agents in dentifrices and mouthwashes for several decades (Healy and Peterson 1999). It is believed that anionic surfactants such as SLS exert their bactericidal action via the cytoplasmic and outer membrane of bacteria by reacting with and solubilising protein moieties resulting in dissolution of the cellular membrane and disaggregation of the cell wall (Gilby and Few 1959; Shafa and Salton 1960; Woldringh and van Iterson 1972; Filip *et al.* 1973). *In vivo* experiments on dentifrices and mouthwashes containing SLS have showed that SLS possesses an anti-plaque effect, even when used singly (Jenkins *et al.* 1991). The same study however, concluded that incorporation of SLS increased the antibacterial activity of other antimicrobials such as triclosan (Jenkins *et al.* 1991). The increased activity of triclosan in the presence of SLS is probably due to

triclosan being dissolved by the hydrophobic interior of SLS micelles until it reaches a lipophilic target were it produces its action (Rolla *et al.* 1993).

3.2 Selection of representative test bacteria for *in-vitro* studies

Continuous contact with environmental oxygen is required for the growth of obligately aerobic species, and also limits the growth of obligately anaerobic species in the oral microflora. Obligately anaerobic species require anaerobiosis (reduced environmental conditions) for their survival and succession in the plaque microbial community. Thus, the degree of oxidation and reduction (redox potential) in different sites of the mouth influence microbial succession and survival. Early bacterial colonisers such as *N. subflava*, *S. mutans* and *S. oralis* utilize oxygen (obligately for *N. subflava*) as the major final electron acceptor and produce CO_2 (Gottschalk 1986), leading to a reduction in the redox potential, producing more favourable conditions for later colonisers to proliferate in the plaque environment. In addition, secondary colonisers may produce fermentation metabolites such as lactic acid, therefore the redox potential in various depths of a biofilm such as plaque is gradually lowered and becomes more suitable for the growth of various species of microorganism with different oxygen tolerance. The increased cell density and thickness in older plaques may produce strictly anaerobic conditions which will affect bacterial metabolism. For example, an increase in the activity of the glycolytic enzymes of *S. mutans* and changes in the pattern of metabolic pathways occurs under anaerobiosis which can result in lower pH values for prolonged periods that are sufficient to cause dental demineralisation (Marsh and Martin 1999). Hence, any shift in the metabolic activities of early or secondary

colonisers may influence the composition or metabolism in the microbial habitat which could be an initiation parameter for dental disease (Marsh and Martin 1999).

3.3 Assessment of cellular respiratory levels

Cell respiration consists of several interconnected pathways through which the oxidation of substrates is catalysed and potential energy is released (Brock *et al.* 1994). Some of this energy may be stored in the form of ATP, prior to being used in energy-requiring cellular activities. Oxidation of substrates occurs by many pathways and three of the major ones will be discussed in this chapter. These pathways are: *i)* Emden-Meyerhof-Parnas pathway (EMP) or glycolysis: this pathway is the most common sequence of reactions used by most bacteria in which sugars such as glucose are catalysed and converted into two pyruvate molecules with the generation of 2 ATP molecules; *ii)* Fermentation: in the absence of oxygen or other external electron acceptors, microorganisms reduce the pyruvate molecules generated by glycolysis to organic acids such as succinic, lactic and acetic acids which also can be further reduced to alcohol; *iii)* The tricarboxylic acid cycle (citric acid cycle) and oxidative phosphorylation: fermentation results in a relatively small amount of energy compared to aerobic respiration. In aerobic conditions, the primary route for the oxidation of sugars in facultative anaerobic microorganisms is via the EMP pathway and the tricarboxylic acid cycle (also known as Krebs cycle) instead of fermentative pathways (Gilbert 1975; Brock *et al.* 1994). During the oxidation of intermediates in the citric acid cycle, electrons are released and transferred to enzymes that contain the coenzyme NAD^+ or FAD and hence they are converted into NADH, FADH respectively. These NADH, FADH molecules transfer the electrons to oxygen

through the activity of the electron transport system (ETS) in the case of aerobic respiration. The net result of respiring one glucose molecule aerobically is the formation of 38 ATP molecules. In spite of the experiments in this chapter being designed so that microorganisms will grow in preferable aerobic conditions (respiration); (Section 2.9), this study also focuses on anaerobic condition pathways that could potentially occur due to shifts of respiratory pathways in the presence of antimicrobial agents. Such pathways include glycolysis/fermentation which involve lactic acid production accounting for the pathogenicity of some oral microorganisms. With respect to oral health, caries are mainly caused by glycolytic production of acids from dietary sugars; these acids especially lactic acid can lower the pH in dental plaque to values below 4, leading to tooth enamel demineralization which can be progressive and irreversible if the acidic environment is sustained. Measuring cellular respiration/fermentation was, therefore considered to be physiologically relevant in this study due to: *i)* the impact of the antimicrobials tested on cellular membranes, which are the functional sites for bacterial respiration and, *ii)* the ability to measure the activity of the glycolytic/fermentative pathway where their metabolites have been shown to act as cariogenic factors.

3.4 Aims

The aim of this chapter was to investigate the mechanism of action and explore the effects of sub-lethal concentrations of a range of antimicrobial agents commonly found in oral hygiene preparations upon bacterial growth and respiration/fermentation pathways of three different bacterial species under oxygen replete conditions. The microorganisms studied (*N. subflava* and *S. oralis*)

were selected because of their ability to utilise oxygen as a final electron acceptor in their metabolic respiratory reactions. *E. coli* on the other hand, was used as a model organism commonly studied as a paradigm bacteria possessing the principle pathways participating in cellular respiration.

3.5 Methods

A quantitative and reproducible method was used to assess both antibacterial action and the effects on respiratory activity pattern produced by sub-lethal levels of antimicrobial agents. Initially, MIC levels were determined for the three species as described in Section 2.6, bacterial growth was measured spectrophotometricaly and optical densities plotted as a function of time. Growth rates were calculated as a percentage of the control slope and plotted against the antimicrobial concentration; this was then extrapolated to the concentration axis (x-axis) in order to determine the MIC. All initial readings for optical densities of the growth curves were normalised to a value of 1, in order to reduce errors in growth patterns that might occur due to changes in the initial concentration of bacteria in the culture (aliquots variance). All starting optical density values were, however, within the range of (±0.19-0.23).

3.6 Results

3.6.1 Determination of growth inhibition by oral antimicrobial agents

3.6.1.1 Effects of exposure of *N. subflava* to antimicrobial agents

Growth curves generated in the microtiter plate reader were used to monitor and investigate the growth of *N. subflava* in the presence of various concentrations of antimicrobial agents, and plotted as a function of time at each concentration tested (Figs. 3.2; 3.3; 3.4; and 3.5). It is important to note that the concentrations tested

for each antimicrobial represented doubling dilutions (for 11 separate concentrations). However, for reasons of clarity, only selected values are given in the graphs. An exception for that was Fig. 3.2 in which all the tested antimicrobial concentrations were shown and stated. The data presented in Figs. 3.2; 3.3; 3.4; and 3.5 show that appropriate concentrations for growth inhibition lay between 24.4µg.ml^{-1} and 48.8µg.ml^{-1} for SLS (Fig. 3.2), between 0.39mg.ml^{-1} and 0.195mg.ml^{-1} for zinc lactate (Fig. 3.3), between 0.8µg.ml^{-1} and 1.6µg.ml^{-1} for triclosan (Fig. 3.4), and for stannous fluoride lay between 0.625mg.ml^{-1} and 1.25mg.ml^{-1} (Fig. 3.5).
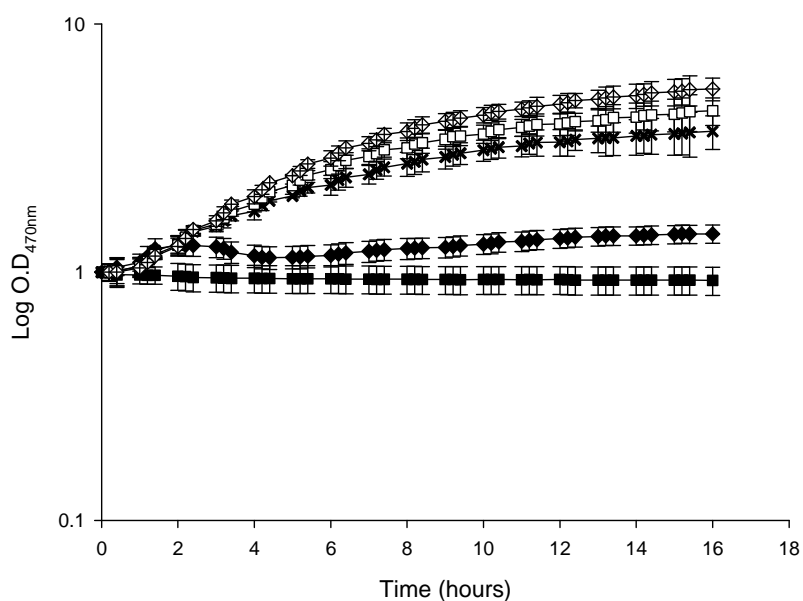
Figure 3.2: Growth curves for *N. subflava* exposed to a range of concentrations of sodium lauryl sulphate, (–●–) 6.25mg.ml^{-1}; (–⊖–) 3.125mg.ml^{-1}; (–★–) 1.56mg.ml^{-1}; (–▼–) 0.78mg.ml^{-1}; (–▽–) 0.39mg.ml^{-1}; (–▲–) 0.195mg.ml^{-1}; (–△–) 97.6µg.ml^{-1}; (–■–) 48.8µg.ml^{-1}; (–◆–) 24.4µg.ml^{-1}; (–✻–) 12.2µg.ml^{-1}; (–□–) 6.1µg.ml^{-1}; (–◈–) control. Points represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3).
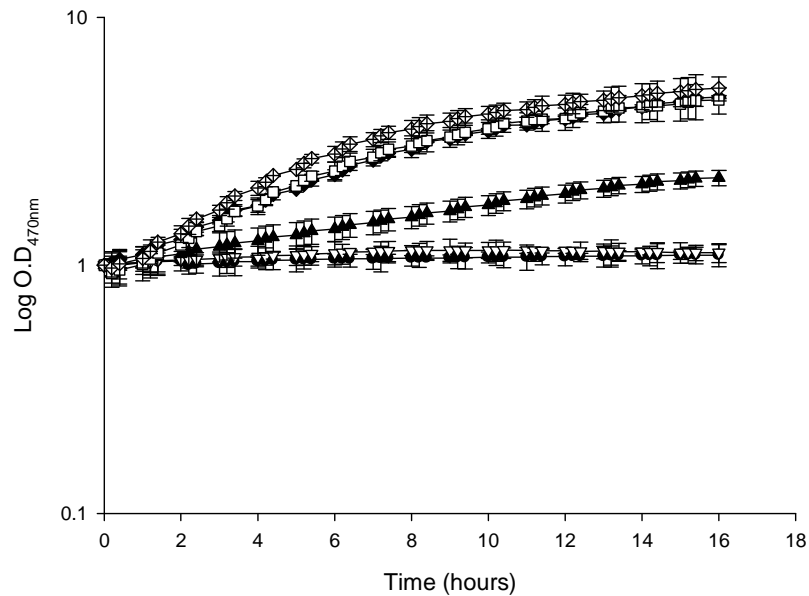
Figure 3.3: Growth curves for *N. subflava* exposed to a range of concentrations of zinc lactate, (–●–) 6.25mg.ml^{-1}; (–▽–) 0.39mg.ml^{-1}; (–▲–) 0.195mg.ml^{-1}; (–◆–) 24.4µg.ml^{-1}; (–□–) 6.1µg.ml^{-1}; (–◇–) control. Points represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3).
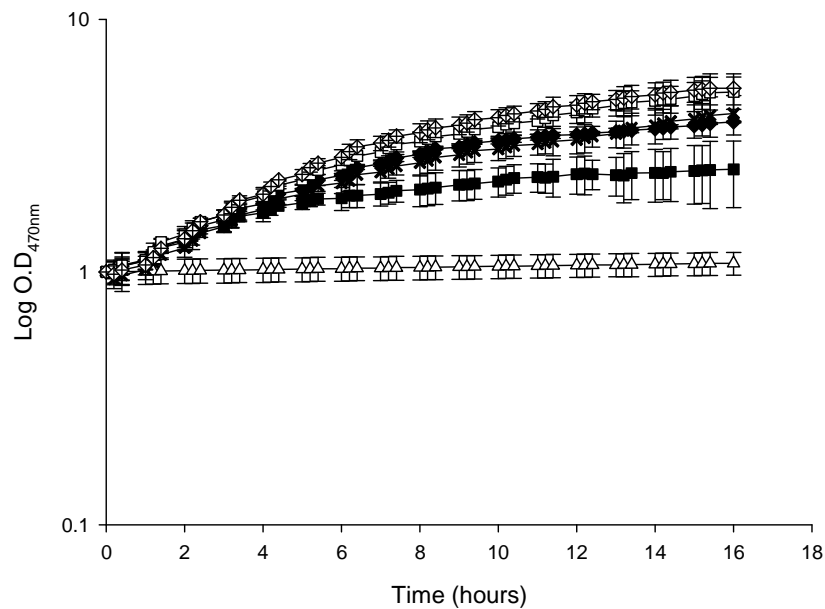
Figure 3.4: Growth curves for *N. subflava* exposed to a range of concentrations of triclosan, (–△–) 1.6µg.ml^{-1}; (–■–) 0.8µg.ml^{-1}; (–◆–) 0.4µg.ml^{-1}; (–✳–) 0.2µg.ml^{-10}; (–□–) DMSO 1%; (–◇–) control. Points represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3).
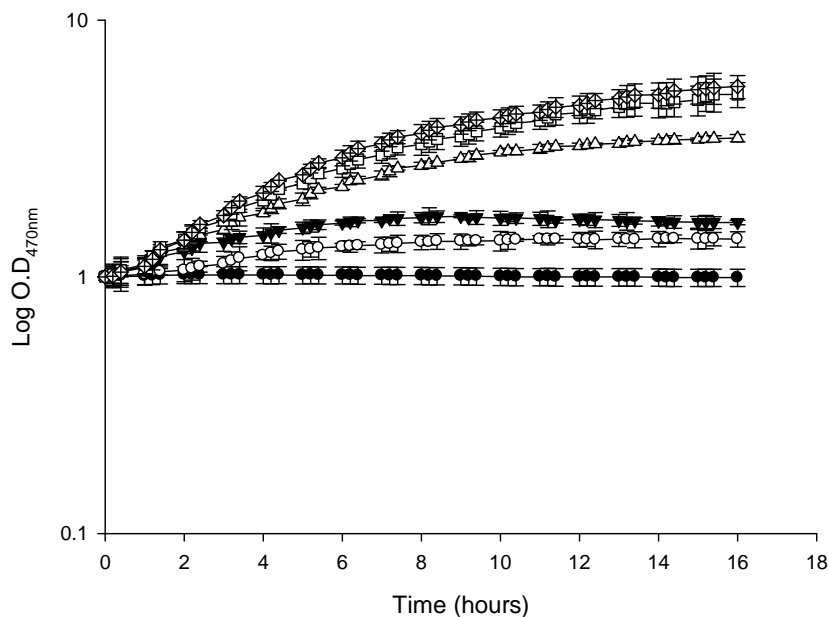
Figure 3.5: Growth curves for *N. subflava* exposed to a range of concentrations of stannous fluoride, (–●–) 1.25mg.ml^{-1}; (–⊖–) 0.625mg.ml^{-1}; (–▼–) 156µg.ml^{-1}; (–△–) 19.5µg.ml^{-1}; (–□–) 1.2µg.ml^{-1}; (–◆–) control. Points represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3).

The same figures also show that the decrease in optical densities is proportional to the increase of the concentration of each antimicrobial. The linear regression from each growth curve was subsequently used to calculate the rate of growth in mid-log phase and expressed as percentage of control values in the presence of various concentrations for each antimicrobial.

The growth rate curves generated were extrapolated to the x-axis (concentration axis) to determine the MIC for each antimicrobial individually (Figs. 3.6; 3.7; 3.8 and 3.9). The MIC values generated from Figs. 3.6; 3.7; 3.8 and 3.9 show that these values were within the range of the MIC values generated from Figs. 3.2; 3.3; 3.4; and 3.5 except for triclosan calculated MIC that was a little bit higher than that of the anticipated MIC as shown in Table 3.1.
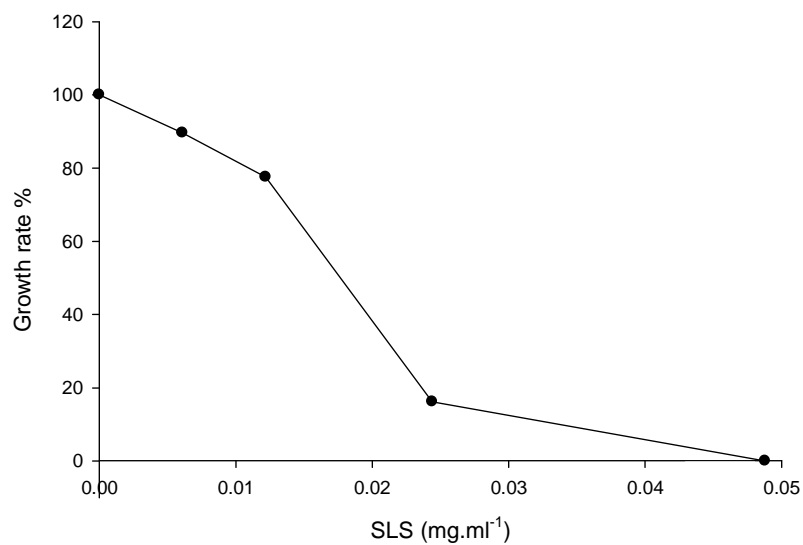
Figure 3.6: Percentage growth rate of *N. subflava* in the presence of sodium lauryl sulphate. Dotted line represents the extrapolation line for MIC calculation.
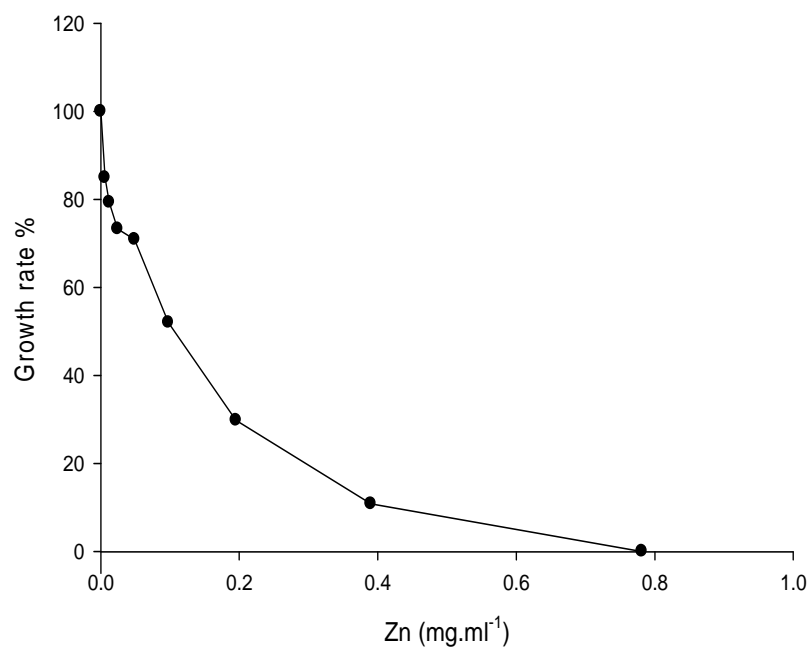
Figure 3.7: Percentage growth rate of *N. subflava* in the presence of zinc lactate. Dotted line represents the extrapolation line for MIC calculation.
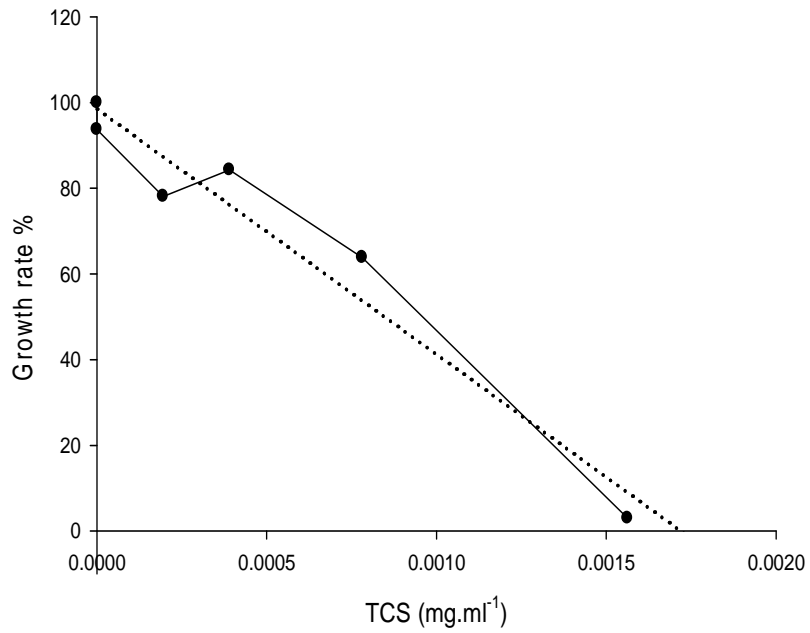
Figure 3.8: Percentage growth rate of *N. subflava* in the presence of triclosan. Dotted line represents the extrapolation line for MIC calculation.
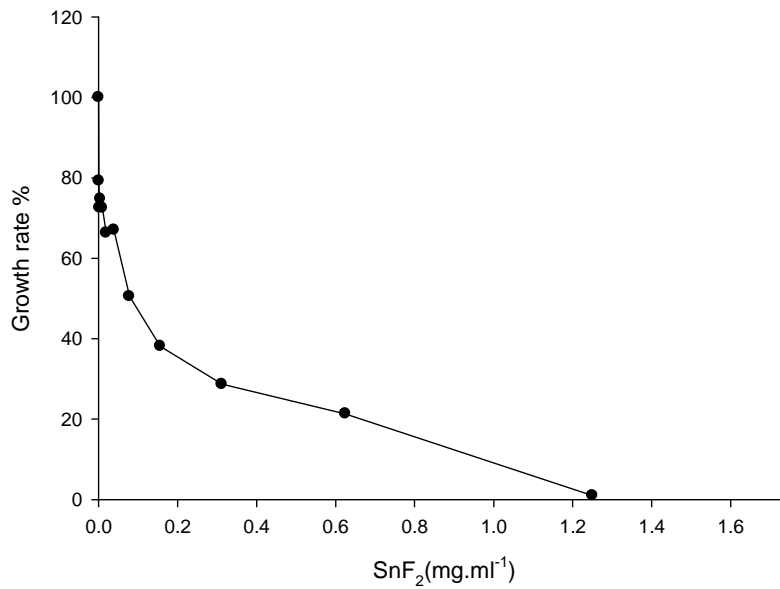
Figure 3.9: Percentage growth rate of *N. subflava* in the presence of stannous fluoride. Dotted line represents the extrapolation line for MIC calculation.

3.6.1.2 Effects of exposure of *S. oralis* to antimicrobial agents

In a similar manner to the data shown in Section 3.6.1.1, data generated in Figs. 3.10-3.17 were used to calculate the MIC of each concentration of different antimicrobials on the growth rate of *S. oralis*. Data in Figure 3.10 show that concentrations of SLS lower than 24.4µg.ml^{-1} had little effect on the growth of *S. oralis* cultures. SLS concentration of 24.4µg.ml^{-1} had an effect on slightly reducing the optical density of bacterial culture at the onset of stationary phase which in turn reduced the yield.
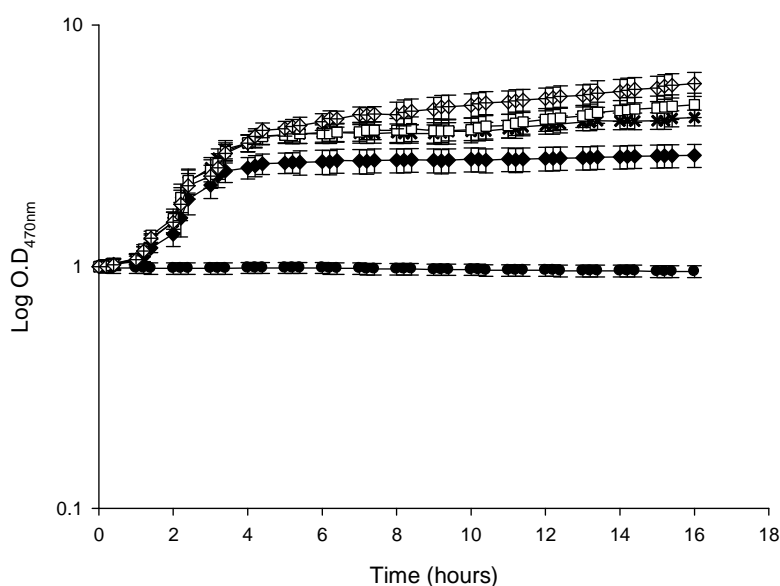
Figure 3.10: Growth curves for *S. oralis* exposed to a range of concentrations of sodium lauryl sulphate, (–•–) 6.25mg.ml^{-1}; (–◆–) 24.4µg.ml^{-1}; (–✳–) 12.2µg.ml^{-1}; (–□–) 6.1µg.ml^{-1}; (–◇–) control. Points represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3).

On the other hand, data in Figs. 3.11, 3.12, and 3.13 showed that concentrations of 0.195mg.ml^{-1} of Zn, 3.12µg.ml^{-1} of TCS and 156µg.ml^{-1} of SnF$_2$ had a greater effect on reducing both the onset of stationary phase and the yield of the microorganisms in the culture.
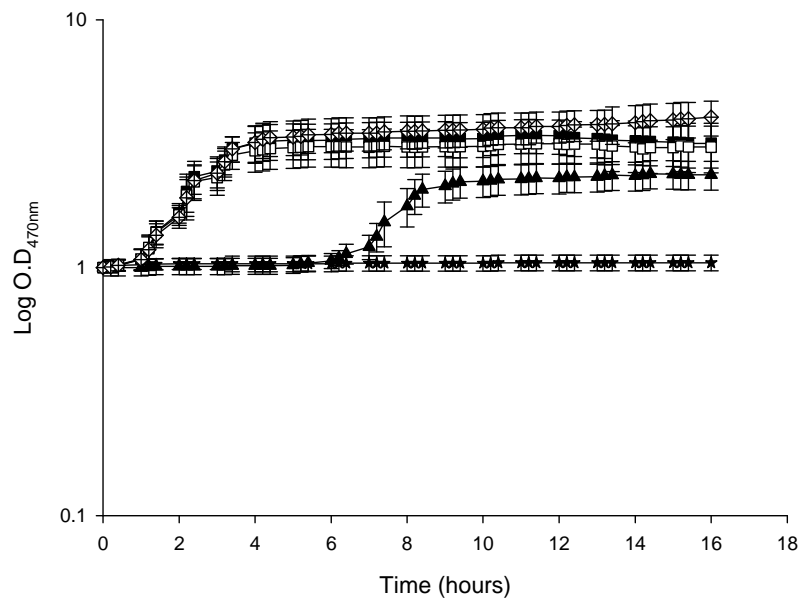
Figure 3.11: Growth curves for *S. oralis* exposed to a range of concentrations of zinc lactate, (—★—) 1.56mg.ml^{-1}; (—▲—) 0.195mg.ml^{-1}; (—■—) 48.8μg.ml^{-1}; (—□—) 6.1μg.ml^{-1}; (—◇—) control. Points represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3).

The MICs of the mentioned antimicrobial agents were generated by extrapolating the growth rate percentage curves to the concentration axis as mentioned in Section 3.5, and are shown in Table 3.1.
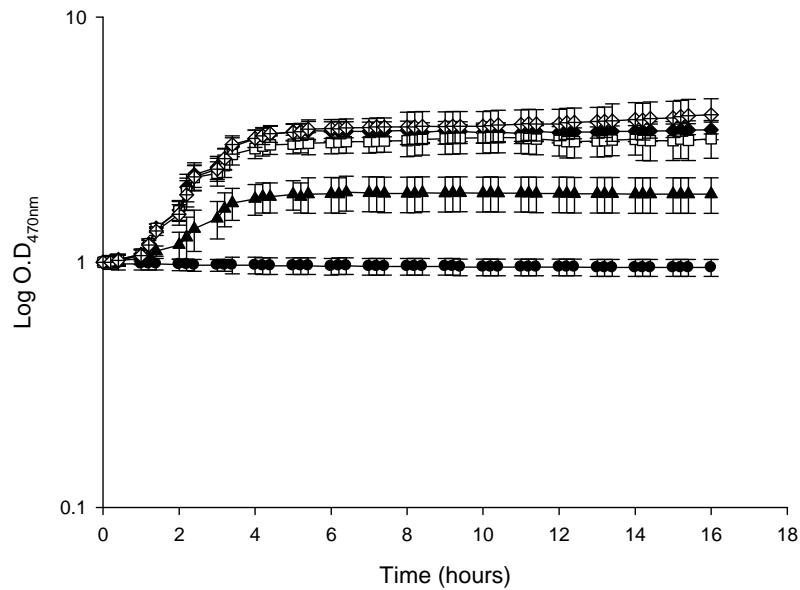
Figure 3.12: Growth curves for *S. oralis* exposed to a range of concentrations of triclosan, (–•–) 100µg.ml^{-1}; (–▲–) 3.12µg.ml^{-1}; (–♦–) 0.4µg.ml^{-1}; (–□–) DMSO 1%; (–◇–) control. Points represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3).
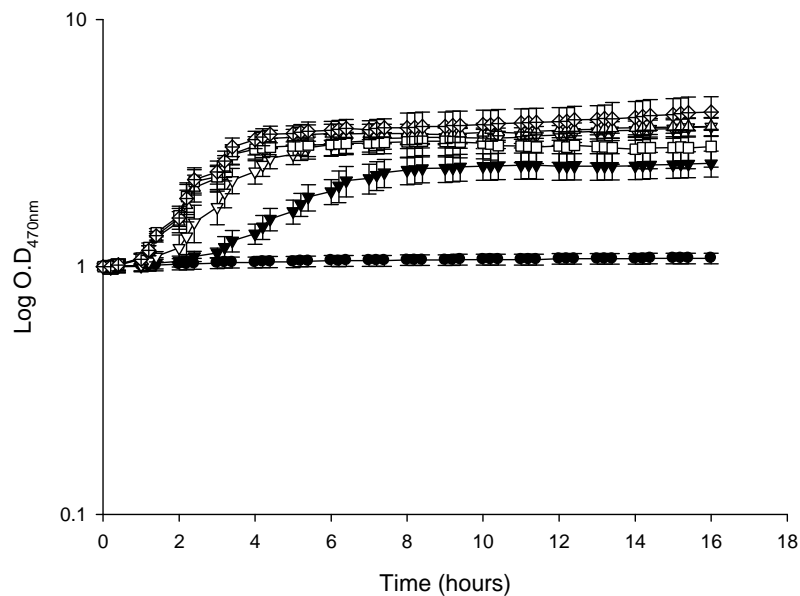
Figure 3.13: Growth curves for *S. oralis* exposed to a range of concentrations of stannous fluoride, (–•–) 1.25mg.ml^{-1}; (–▼–) 156µg.ml^{-1}; (–▽–) 78.0µg.ml^{-1}; (–△–) 19.5µg.ml^{-1}; (–□–) 1.2µg.ml^{-1}; (–◇–) control. Points represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3).
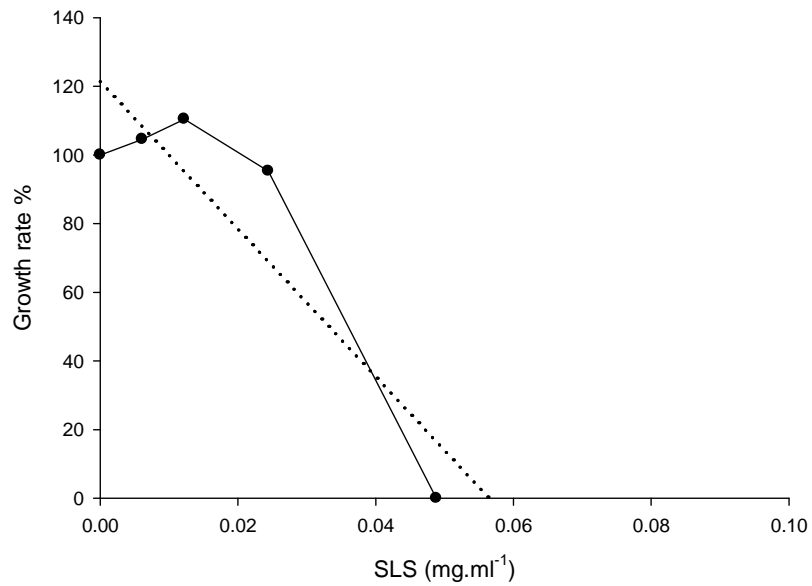
Figure 3.14: Percentage growth rate of *S. oralis* in the presence of sodium lauryl sulphate**.** Dotted line represents the extrapolation line for MIC calculation.
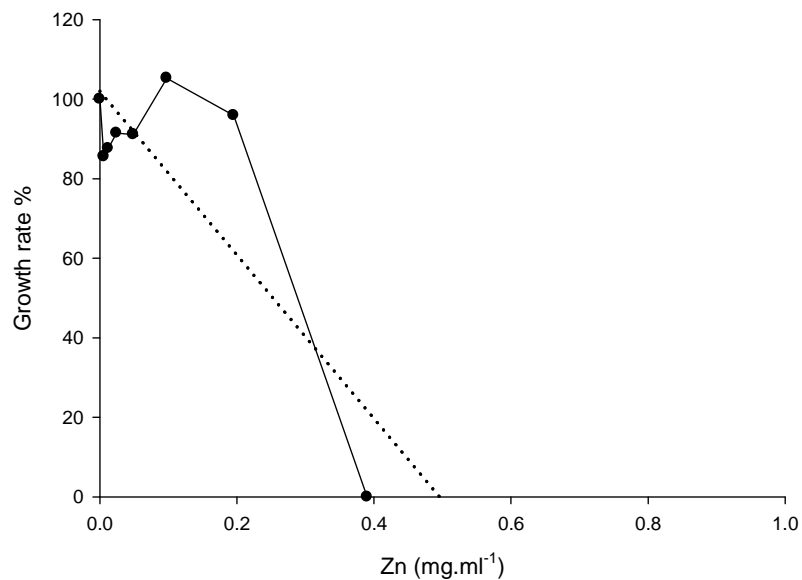
Figure 3.15: Percentage growth rate of *S. oralis* in the presence of zinc lactate. Dotted line represents the extrapolation line for MIC calculation.
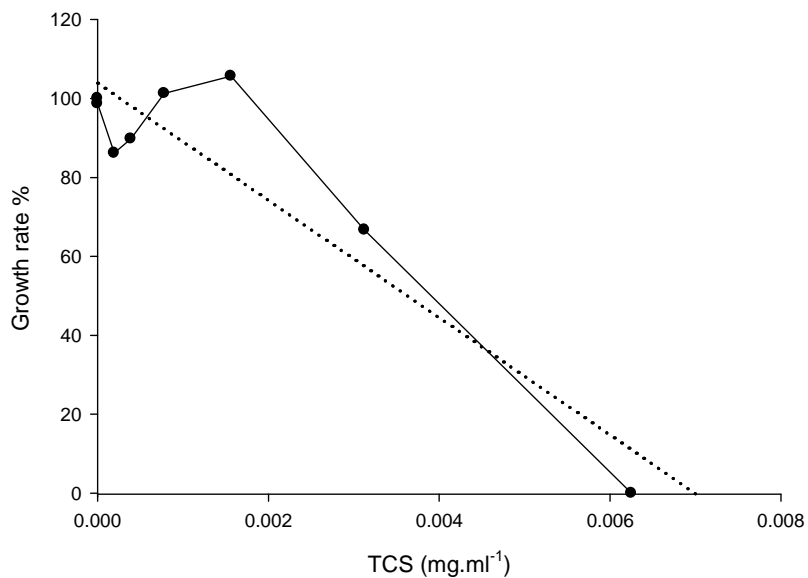
Figure 3.16: Percentage growth rate of *S. oralis* in the presence of triclosan. Dotted line represents the extrapolation line for MIC calculation.
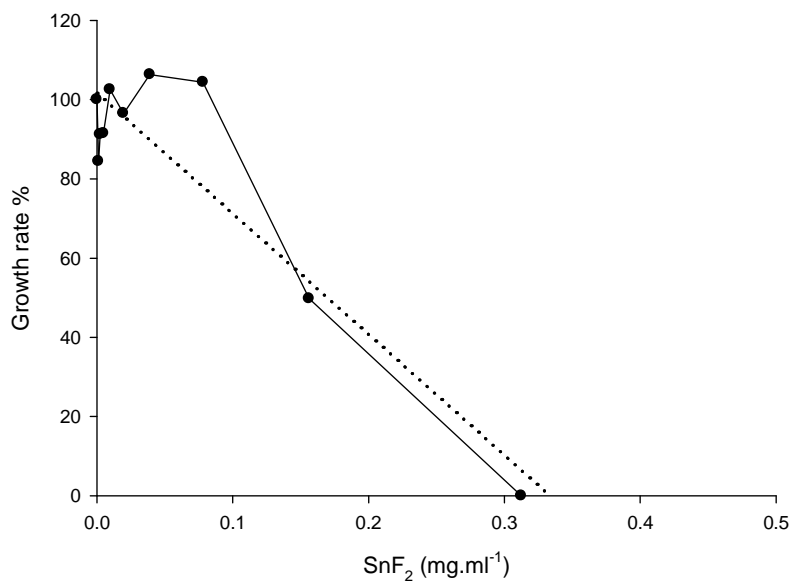
Figure 3.17: Percentage growth rate of *S. oralis* in the presence of stannous fluoride. Dotted line represents the extrapolation line for MIC calculation.

3.6.1.3 Effects of exposure of *E. coli* to antimicrobial agents

Growth curves presented in Fig. 3.18 show that various concentrations of SLS could only cause a slight inhibition in the yield of *E. coli*. This was only determined at 6.25mg.ml^{-1} of SLS.
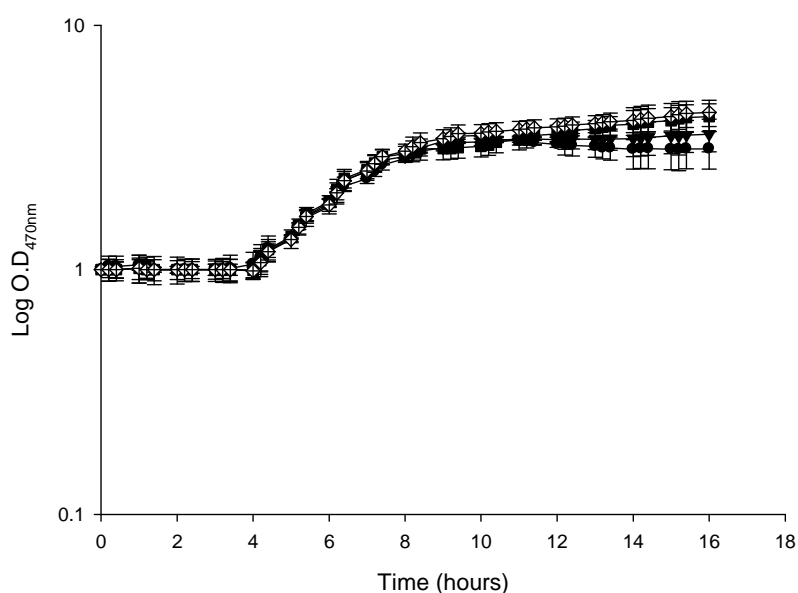
Figure 3.18: Growth curves for *E. coli* exposed to a range of concentrations of sodium lauryl sulphate, (–●–) 6.25mg.ml^{-1}; (–▼–) 0.78mg.ml^{-1}; (–■–) 48.8μg.ml^{-1}; (–♦–) 24.4μg.ml^{-1}; (–◇–) control. Points represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3).

Fig. 3.19 show that concentrations of 0.39mg.ml^{-1} and higher were required to cause a reduction in both the onset of stationary phase and the yield of *E. coli*, whilst concentrations of 3.12μg.ml^{-1} and higher of TCS were required to produce the same effects on the bacterial cultures (Fig. 3.20). SnF$_2$ levels showed no effects on the growth of *E. coli* cultures except in the presence 1.25mg.ml^{-1} at which both the onset of stationary phase and the yield were reduced as shown in Fig. 3.21. The MIC levels of Zn, TCS and SnF$_2$ were achieved from the

extrapolation of the growth rate percentage curves of *E.coli* (Figs. 3.19; 3.20 and

3.21), whilst no MIC was detected in Fig. 3.18.
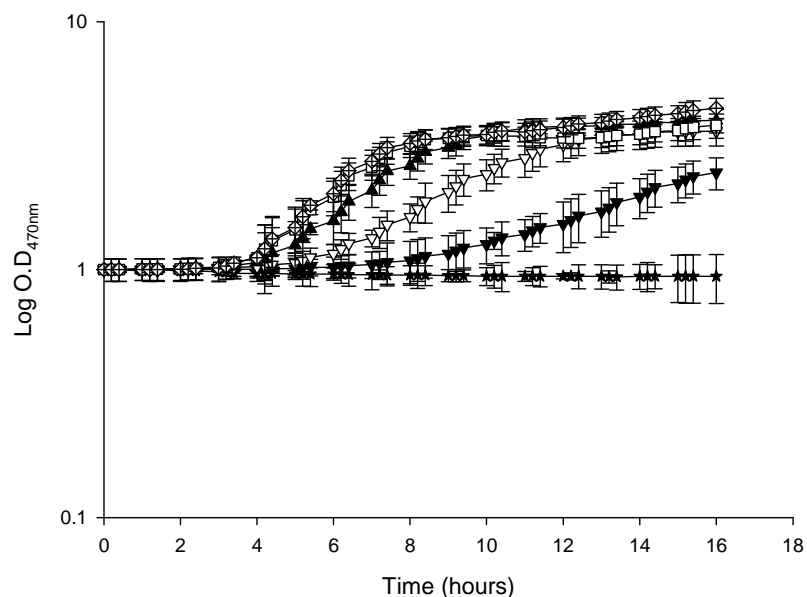
Figure 3.19: Growth curves for *E. coli* exposed to a range of concentrations of zinc lactate, (—★—) 1.56mg.ml^{-1}; (—▼—) 0.78mg.ml^{-1}; (—▽—) 0.39mg.ml^{-1}; (—▲—) 0.195mg.ml^{-1} ; (—□—) 6.1μg.ml^{-1}; (—◇—) control. Points represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3).
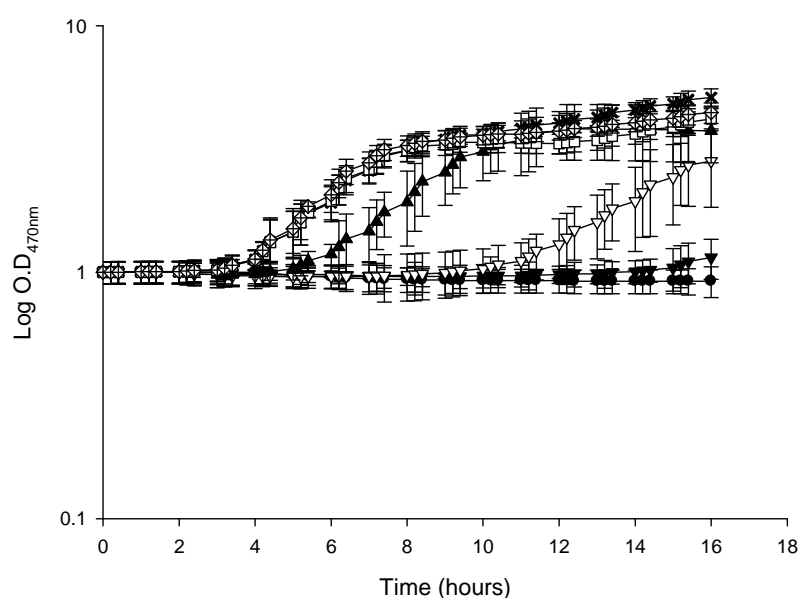
Figure 3.20: Growth curves for *E. coli* exposed to a range of concentrations of triclosan, (—●—) 100μg.ml^{-1}; (—▼—) 12.5μg.ml^{-1}; (—▽—) 6.25μg.ml^{-1}; (—▲—) 3.12μg.ml^{-1}; (—✶—) 0.2μg.ml^{-1}; (—□—) DMSO 1%; (—◇—) control. Points represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3).
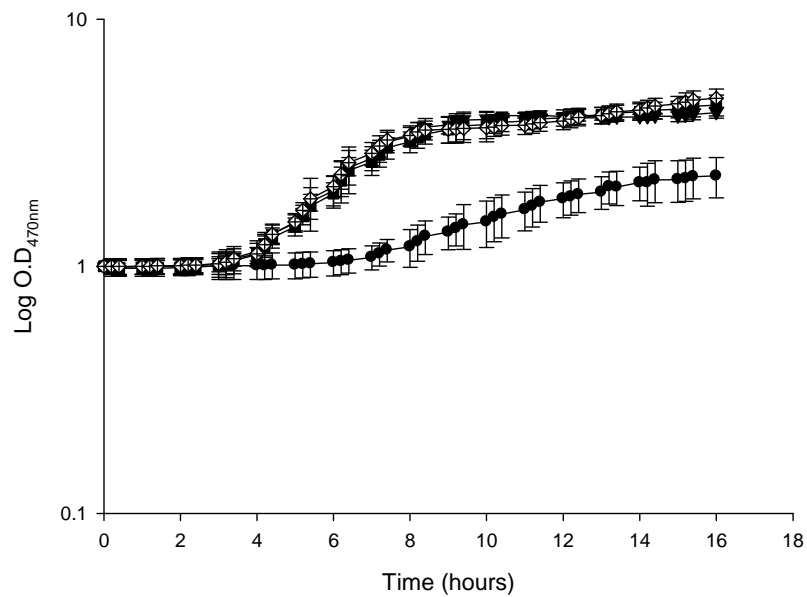
Figure 3.21: Growth curves for *E. coli* exposed to a range of concentrations of stannous fluoride, (—•—) 1.25mg.ml^{-1}; (—▼—) 156μg.ml^{-1}; (—■—) 9.75μg.ml^{-1}; (—◇—) control. Points represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3).

The MIC ranges of the different antimicrobials used in this study are shown in Table 3.1, in which these ranges are compared with the levels of the same molecules present in their counterpart oral hygiene products. The growth curve anticipated MIC ranges were visually selected by detecting the antimicrobial concentration values of the growth curve showing the lowest growth activity and the nearest growth curve with total growth inhibition. It is clear from the values listed in the table that most of the values of the growth curve anticipated MIC ranges were within the range of the MIC levels that were detected from the extrapolation of the percentage growth rate curves of the tested bacterial species. It is also clear that both the anticipated and calculated antimicrobial values are lower than the concentrations of the oral hygiene antimicrobial counterparts except for SLS test results where the tested concentration had minimal effect on the growth of *E. coli* species.
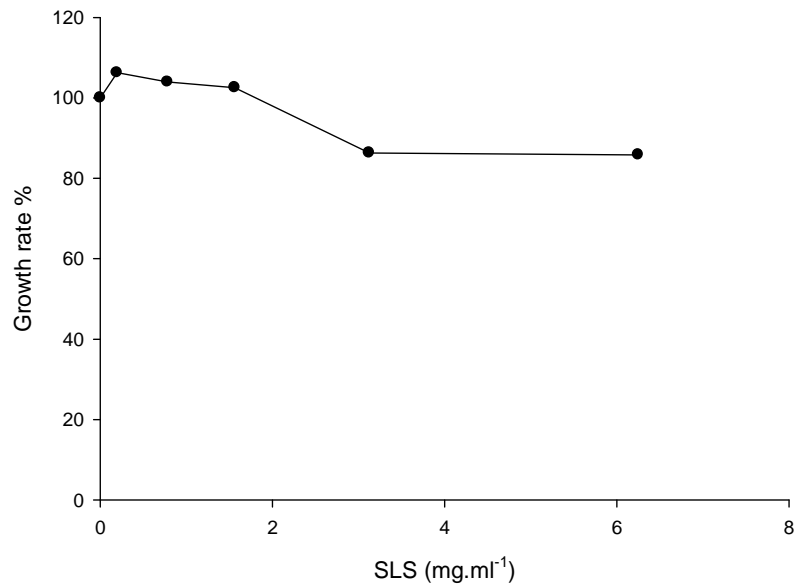
Figure 3.22: Percentage growth rate of *E. coli* in the presence of sodium lauryl sulphate.
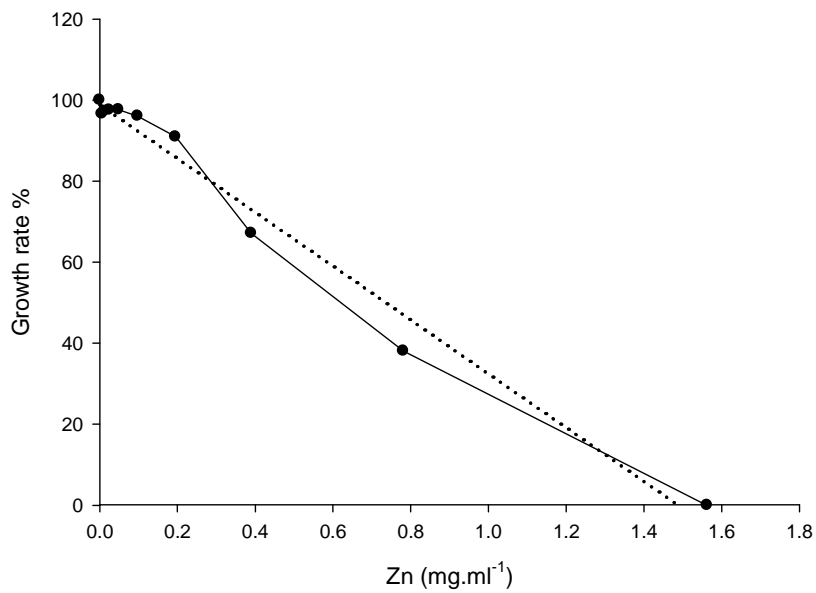
Figure 3.23: Percentage growth rate of *E. coli* in the presence of zinc lactate. Dotted line represents the extrapolation line for MIC calculation.
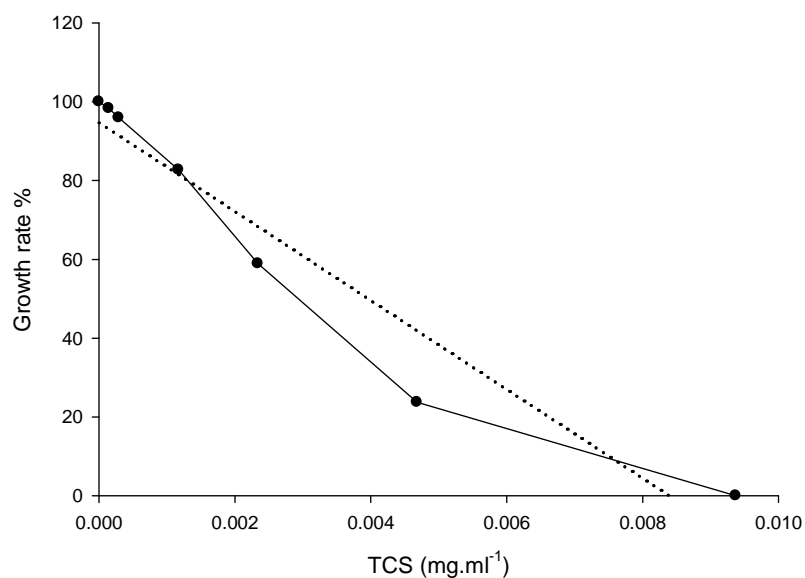
Figure 3.24: Percentage growth rate of *E. coli* in the presence of triclosan. Dotted line represents the extrapolation line for MIC calculation.
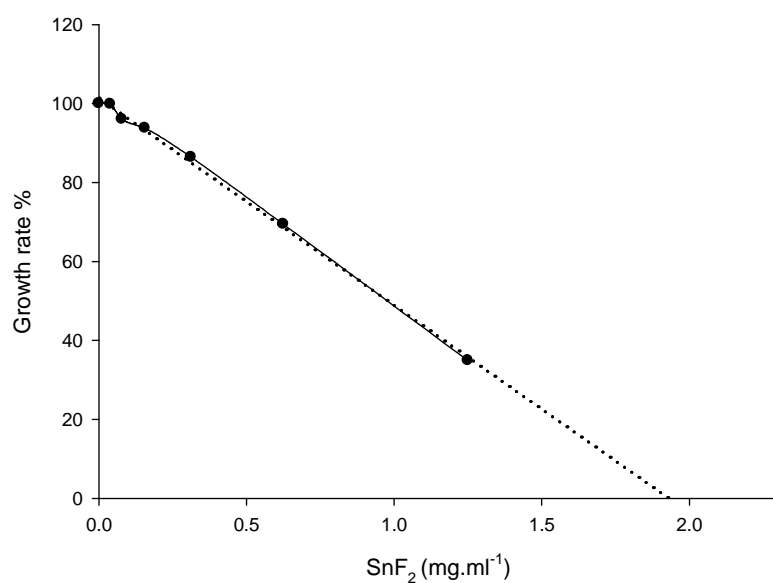
Figure 3.25: Percentage growth rate of *E. coli* in the presence of stannous fluoride. Dotted line represents the extrapolation line for MIC calculation.

Table 3.1: MIC levels of different antimicrobial agents against *N. subflava*, *S. oralis* and *E. coli*.

Micro-organisms	Growth curve anticipated MIC range (mg.ml^{-1})				Calculated MIC (mg.ml^{-1})				Oral hygiene antimicrobial concentration (mg.ml^{-1})			
	SLS	Zn	TCS	SnF$_2$	SLS	Zn	TCS	SnF$_2$	SLS	Zn	TCS	SnF$_2$
N. subflava	24.4-48.8[*]	0.39-0.78	0.8-1.6[*]	0.63-1.25	49[*]	0.78	1.7[*]	1.25				
S. oralis	24.4-48.8[*]	0.20-0.39	3.12-6.25[*]	0.16-0.32	49[*]	0.39	6.20[*]	0.31	10	10	3.0	4.5
E. coli	ND[**]	0.78-1.56	12.5-25[*]	> 1.25	ND	1.58	9.5[*]	1.85				

[*] Concentrations in µg.ml^{-1}, [**] Not determined.

3.6.2 Assessment of bacterial respiratory activity

Tetrazolium salts have been used in many previous studies as indicators of bacterial respiratory activity (Roslev and King 1993; Smith and McFeters 1997; Bhupathiraju *et al.* 1999). These dyes serve as artificial electron acceptors for different metabolic chain reactions. Initially tetrazolium salts are colourless in solution but they become coloured when they are reduced by the action of different enzymes in the respiratory system of microorganisms. Reduction of tetrazolium salts with different redox potentials results in the formation of coloured, insoluble, intracellular formazones crystals (Smith and McFeters 1997). In this chapter, bacterial respiratory pathways were assessed at different points using two types of tetrazolium salts with different redox potentials. The site of reduction of TTC has been proven to be at the level of electron transport chain specifically at cytochrome c oxidase and it is believed that it competes with molecular oxygen for reducing equivalents (Slater and Sawyer 1963; Gilbert 1975; Bochner and Savageau 1976; Dorthu *et al.* 1992). On the other hand, the reduction of INT is coupled to the reduction of NAD(P)H-dependant dehydrogenases (Babson and Babson 1973; Gilbert 1975; Smith and McFeters 1997). Therefore, INT reduction allows the detection of glycolysis, fermentation, TCA and ETS pathways whilst TTC reduction is strictly an identification of the ETS chain.

3.6.2.1 Bacterial respiration assessment after long-term (18 hours) exposure to different antimicrobial agents

The respiratory potentials of *N. subflava, S. sanguis and E. coli* were assessed for one hour after 16 hours exposure to sub-MIC levels of different antimicrobial agents (Section 2.8). Incubation of tetrazolium salts with microorganisms for more than one hour may cause abiotic reduction of the dye rather than enzymatic reduction (data not shown). Bacterial respiration was monitored spectrophotometricaly. Validation studies showed that the optimal wavelength for measuring tetrazolium salts activity (INT & TTC) was 550 nm. Optical densities for each reduced dye were plotted as a function of time and the oxidation/reduction rate was calculated as a percentage of the control slope and plotted against the antimicrobial concentration.

3.6.2.1.1 Assessment of *N. subflava* respiration activity

Tetrazolium salts assays of *N. subflava* were used to assess the respiratory potential in the presence of sub-lethal concentrations of various antimicrobials. Data shown in Figs. 3.26 and 3.27 demonstrate the ability of bacterial cells to respire in the presence of INT and TTC respectively with SLS as the antimicrobial agent. Data presented in Fig. 3.26 show that the increase in the optical density is proportional to the decrease in the concentration of SLS, whereas data presented in Fig. 3.27 show that cells exposed to sub-lethal concentrations of SLS respire at a much higher rate than the control cells. The results presented in Figs. 3.28 and 3.29 also indicate a proportional increase in the optical densities of both INT and TTC respectively with the decrease in the concentration of zinc lactate i,e both tetrazoliums are equally reduced and the increase in the reduction is proportional to the decrease in the concentration of zinc lactate, this inverse proportionality

was also shown with INT and TTC reduction in the presence of stannous fluoride

(Figs. 3.32 and 3.33). Data presented in Fig. 3.31 show a similar respiration

pattern of that in Figs. 3.26 and 3.27 in which the rate of respiration of *N. subflava*

cultures in the presence of high concentrations of triclosan is much higher than

control cultures. This increase in the optical density refers to the increase in the

reduction of TTC in the presence of SLS or triclosan reflects an uncoupling action

of these biocides.
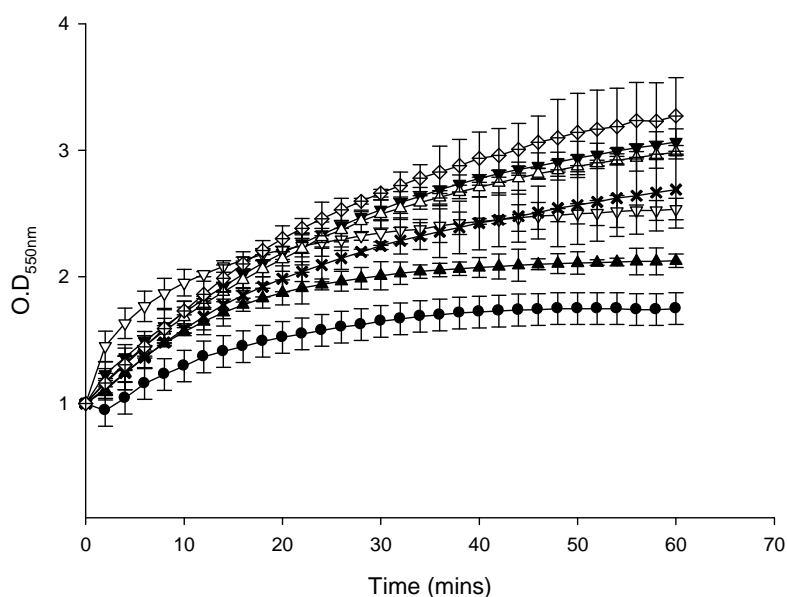
Figure 3.26: The effect of different concentrations of sodium lauryl sulphate on INT activities in *N. subflava*; (—•—) 6.25mg.ml^{-1}; (—▼—) 0.78mg.ml^{-1}; (—▽—) 0.39mg.ml^{-1}; (—▲—) 0.195mg.ml^{-1}; (—△—) 97.6μg.ml^{-1}; (—✳—) 12.2μg.ml^{-1}; (—◇—) control. Points represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3).
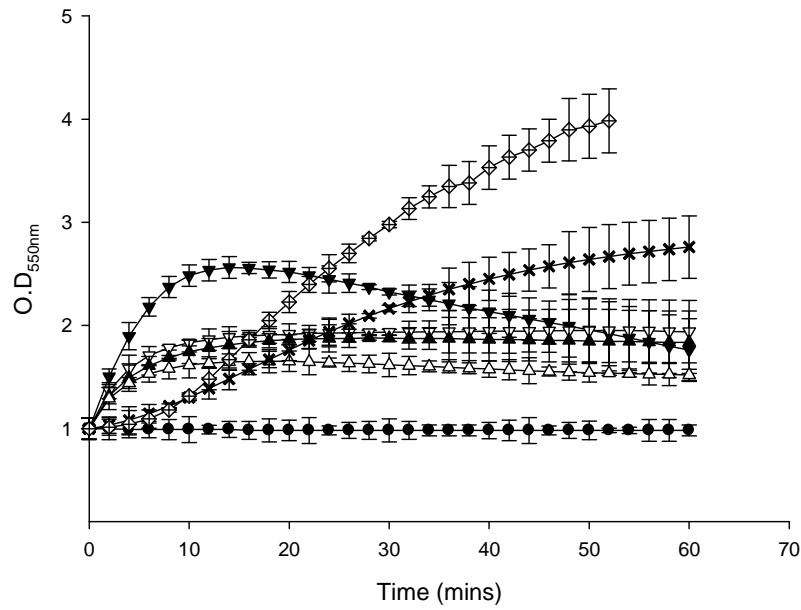
Figure 3.27: The effect of different concentrations of sodium lauryl sulphate on TTC activities in *N. subflava*; (–•–) 6.25mg.ml^{-1}; (–▼–) 0.78mg.ml^{-1}; (–▽–) 0.39mg.ml^{-1} (–▲–) 0.195mg.ml^{-1}; (–△–) 97.6µg.ml^{-1}; (–✳–) 12.2µg.ml^{-1}; (–◈–) control. Points represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3).
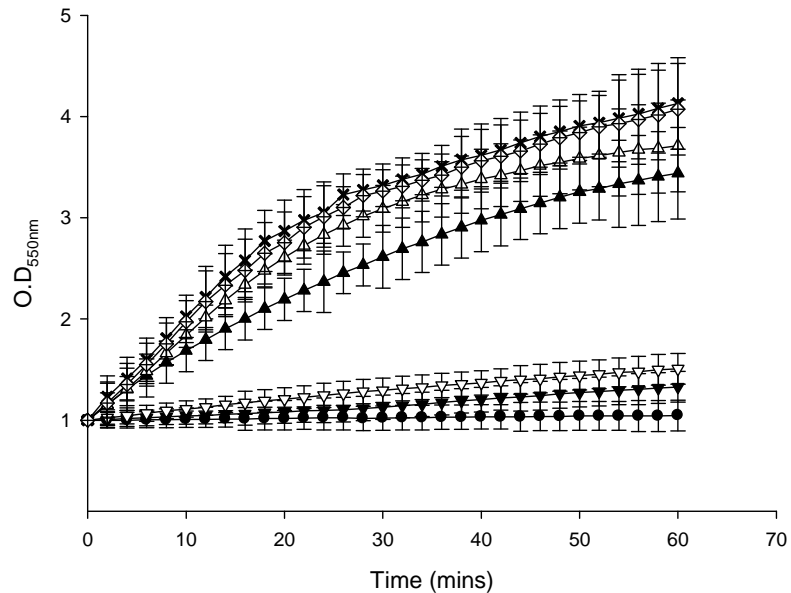
Figure 3.28: The effect of different concentrations of zinc lactate on INT activities in *N. subflava*; (–•–) 6.25mg.ml^{-1}; (–▼–) 0.78mg.ml^{-1}; (–▽–) 0.39mg.ml^{-1}; (–▲–) 0.195mg.ml^{-1}; (–△–) 97.6µg.ml^{-1}; (–✳–) 12.2µg.ml^{-1}; (–◈–) control. Points represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3).
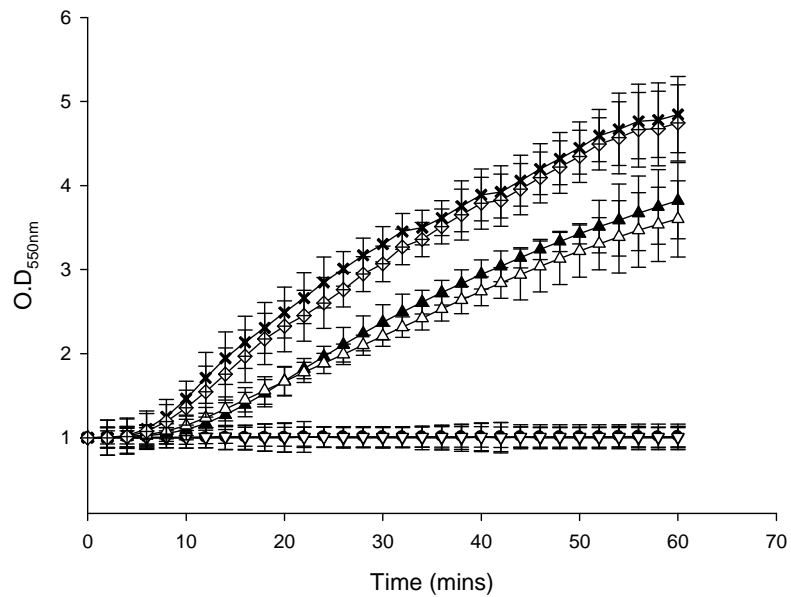
Figure 3.29: The effect of different concentrations of zinc lactate on TTC activities in *N. subflava*; (–●–) 6.25mg.ml^{-1}; (–▼–) 0.78mg.ml^{-1}; (–▽–) 0.39mg.ml^{-1} (–▲–) 0.195mg.ml^{-1}; (–△–) 97.6µg.ml^{-1}; (–✕–) 12.2µg.ml^{-1}; (–◈–) control. Points represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3).

Figure 3.30: The effect of different concentrations of triclosan on INT activities in *N. subflava*; (–●–) 100µg.ml^{-1}; (–○–) 50.0µg.ml^{-1}; (–✳–) 25.0µg.ml^{-1}; (–▼–) 12.5µg.ml^{-1}; (–▽–) 6.25µg.ml^{-1}; (–▲–) 3.12µg.ml^{-1}; (–✕–) 0.2µg.ml^{-1}; (–◈–) control. Points represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3).

Figure 3.31: The effect of different concentrations of triclosan on TTC activities in *N. subflava*; (–•–) 100µg.ml^{-1}; (–⊖–) 50.0µg.ml^{-1}; (–⋆–) 25.0µg.ml^{-1}; (–▼–) 12.5µg.ml^{-1}; (–▽–) 6.25µg.ml^{-1}; (–▲–) 3.12µg.ml^{-1}; (–✻–) 0.2µg.ml^{-1}; (–◇–) control. Points represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3).
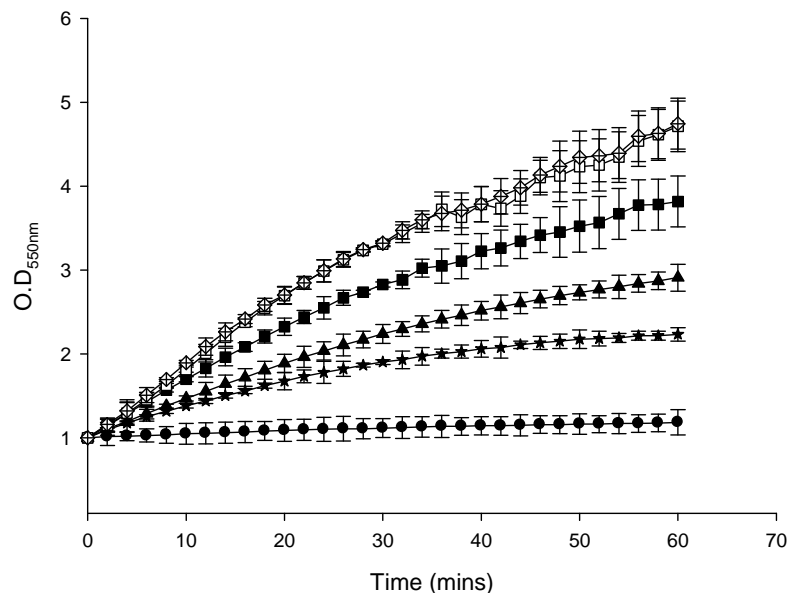
Figure 3.32: The effect of different concentrations of stannous fluoride on INT activities in *N. subflava*; (–•–) 1.25mg.ml^{-1}; (–⋆–) 0.31mg.ml^{-1}; (–▲–) 39.0µg.ml^{-1}; (–■–) 9.75µg.ml^{-1}; (–□–) 1.2µg.ml^{-1}; (–◇–) control. Points represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3).
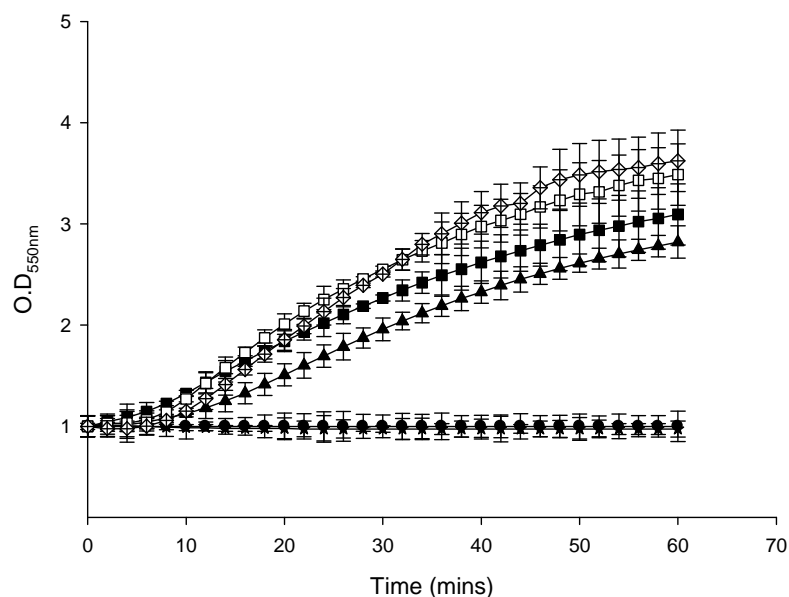
Figure 3.33: The effect of different concentrations of stannous fluoride on TTC activities in *N. subflava*; (–●–) 1.25mg.ml^{-1}; (–★–) 0.31mg.ml^{-1}; (–▲–) 39.0µg.ml^{-1}; (–■–) 9.75µg.ml^{-1}; (–□–) 1.2µg.ml^{-1}; (–◇–) control. Points represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3).

Uncoupling agents remove or degrade the link between the oxidation of coenzymes such as NADH and the phosphorylation of ADP to ATP in the proton motive force. This uncoupling effect results from the ability of compounds such as triclosan and SLS to perturb and damage the cytoplasmic membrane, whereby the cell membrane becomes permeable to external protons resulting in short circuiting the ETS chain and the inhibition of ATP synthesis. As a consequence, the damaged cell needs to respire at a faster rate to survive the injury produced by the uncouplers. Another theory involving the uncoupling effect states that these compounds are highly lipophilic that can pass across the cell membrane and acting as external protons thus also resulting in short circuiting the ETS chain and the inhibition of ATP synthesis (Russell 1992). The linear regression from each reduction curve in Figs. 3.26 to 3.33 was used to calculate the rate of reduction of each tetrazolium salt separately and then expressed as percentage of control

values in the presence of various concentrations of different antimicrobial agents. The increased rate of respiration in Fig. 3.27 can be determined by the increase in the reduction rate of TTC rather than INT in Fig. 3.34 due to the redox potential of TTC at the ETS. Cellular respiration was also increased in the presence of TCS, as shown clearly in Fig. 3.36 where the percentage reduction rate of TTC was clearly higher than INT reduction rate in the presence of various concentrations of TCS. Data presented in Figs. 3.35 and 3.37 represent the percentage reduction rate of both tested tetrazoliums with *N. subflava* cultures in the presence of zinc lactate and stannous fluoride respectively, where the percentage reduction rate of INT and TTC is approximately the same. The equal reduction of tetrazolium salts explains that INT and TTC might be equally reduced at the ETS.
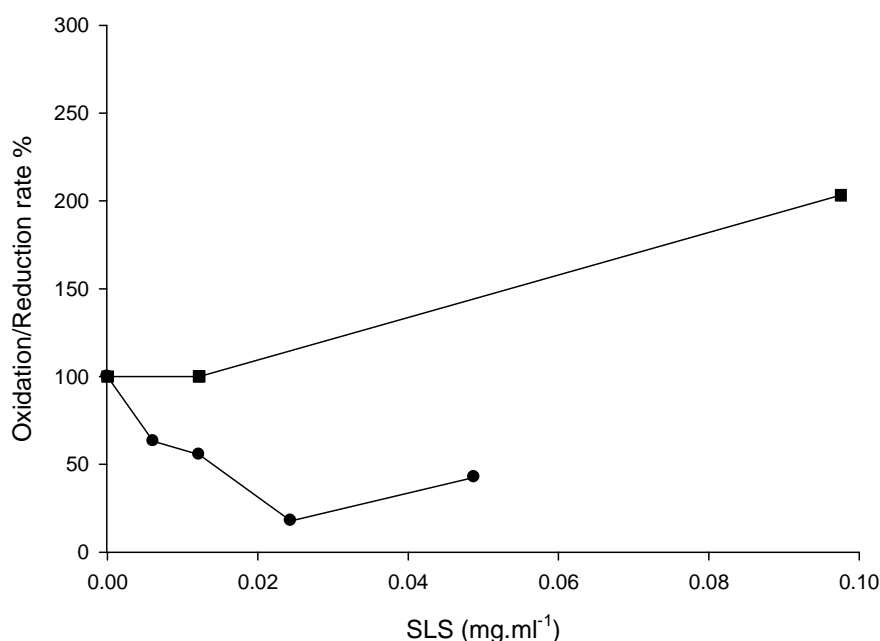
Figure 3.34: The percentage reduction rate of (–■–) TTC and (–●–) INT with *N. subflava* in the presence of sodium lauryl sulphate.
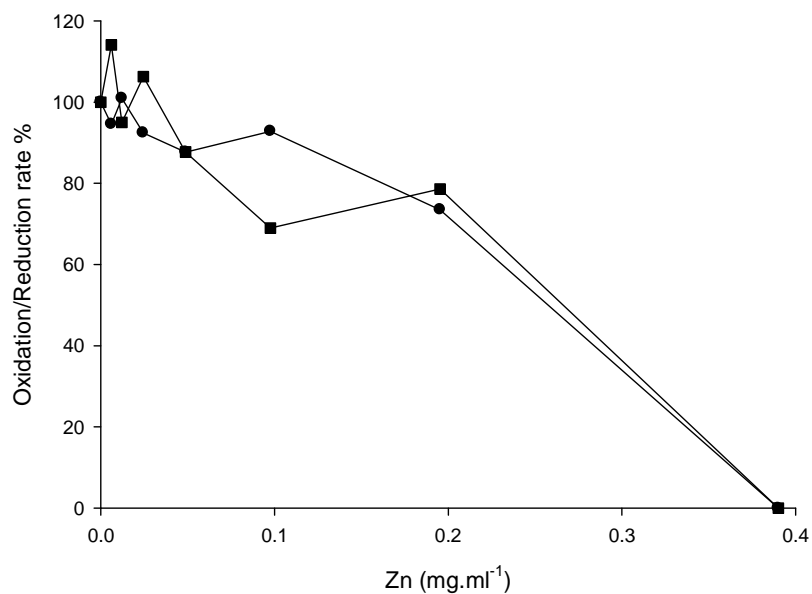
Figure 3.35: The percentage reduction rate of (─■─) TTC and (─●─) INT with *N. subflava* in the presence of zinc lactate.
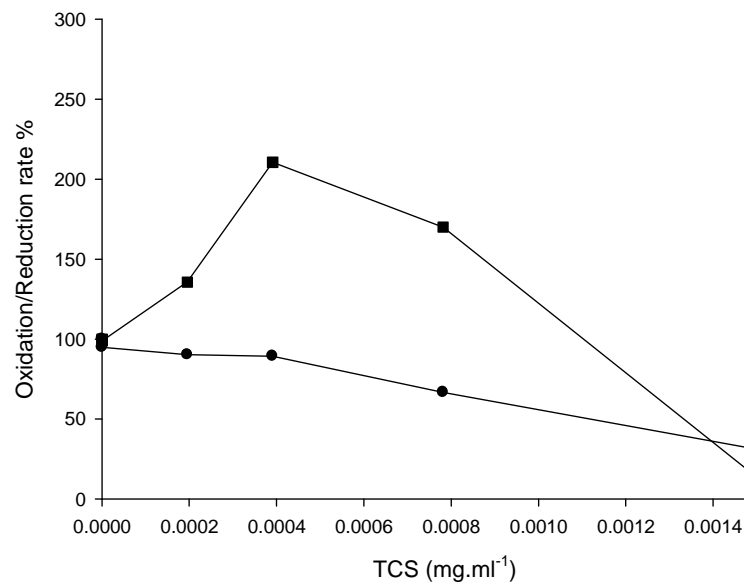
Figure 3.36: The percentage reduction rate of (─■─) TTC and (─●─) INT with *N. subflava* in the presence of triclosan.
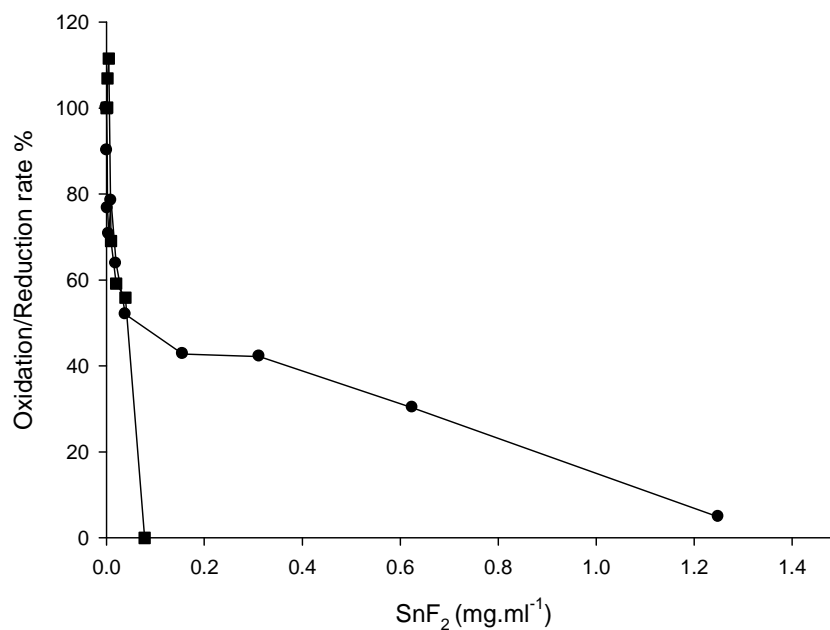
Figure 3.37: The percentage reduction rate of (–■–) TTC and (–●–) INT with *N. subflava* in the presence of stannous fluoride.

3.6.2.1.2 Assessment of *S. oralis* respiration activity

According to the data presented in Figs. 3.38 to 3.45, control cultures of *S. oralis* showed higher optical densities in the reduction of INT as an artificial electron acceptor compared to the optical densities of TTC control cultures. Results also indicated that the addition of 2% glucose had very little effect on the respiratory activity of control cells of *S. oralis* compared to the respiratory activity of *N. subflava* and *E. coli* (section 3.4.2.1.3).
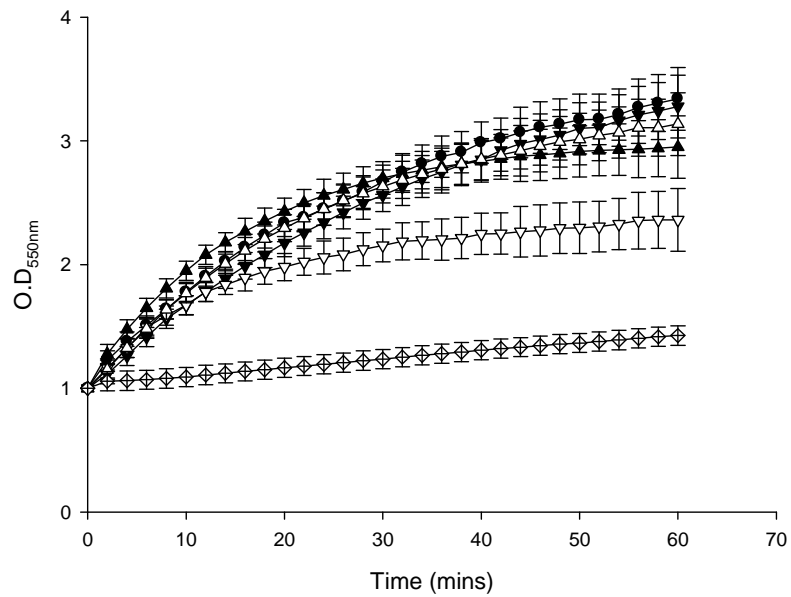
Figure 3.38: The effect of different concentrations of sodium lauryl sulphate on INT activities of *S. oralis*; (–●–) 6.25mg.ml^{-1}; (–▼–) 0.78mg.ml^{-1}; (–▽–) 0.39mg.ml^{-1}; (–▲–) 0.195mg.ml^{-1}; (–△–) 97.6µg.ml^{-1}; (–◇–) control. Points represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3).
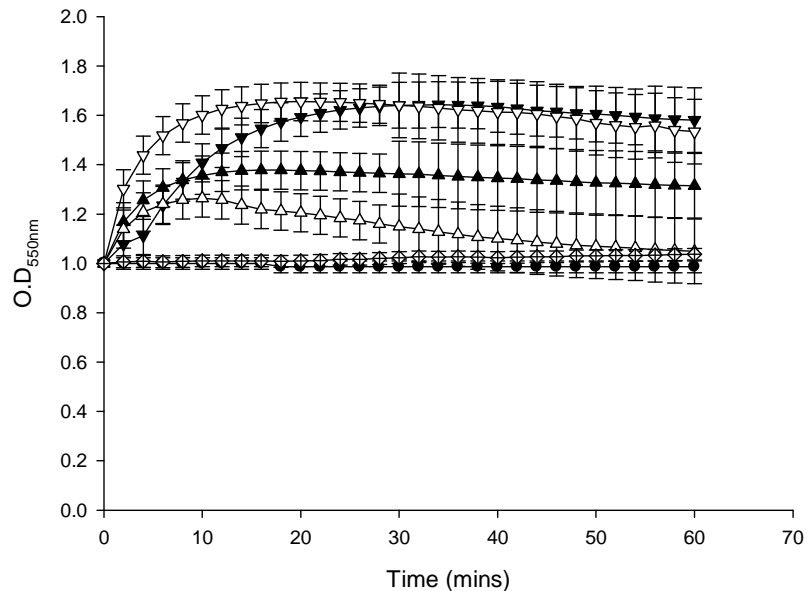
**Figure 3.39:** The effect of different concentrations of sodium lauryl sulphate on TTC activities of *S. oralis*; (–●–) 6.25mg.ml^{-1}; (–▼–) 0.78mg.ml^{-1}; (–▽–) 0.39mg.ml^{-1}; (–▲–) 0.195mg.ml^{-1}; (–△–) 97.6µg.ml^{-1}; (–◇–) control. Points represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3).

114
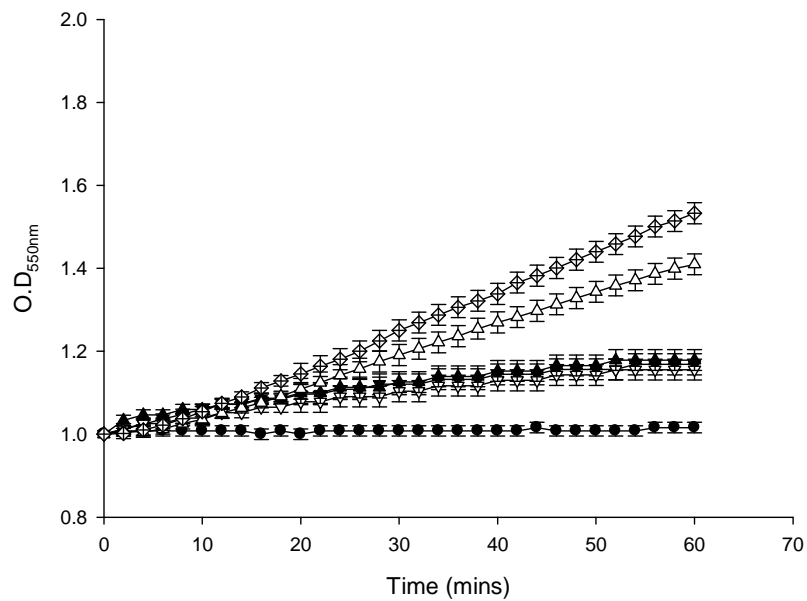
Figure 3.40: The effect of different concentrations of zinc lactate on INT activities of *S. oralis*; (–•–) 6.25mg.ml^{-1}; (–▾–) 0.78mg.ml^{-1}; (–▽–) 0.39mg.ml^{-1}; (–▴–) 0.195mg.ml^{-1}; (–△–) 97.6µg.ml^{-1}; (–◇–) control. Points represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3).
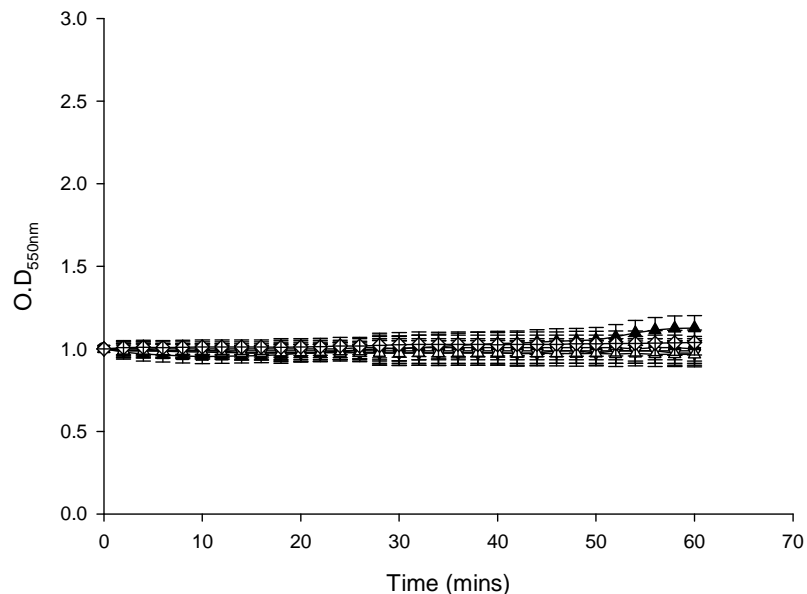
Figure 3.41: The effect of different concentrations of zinc lactate on TTC activities of *S. oralis*; (–•–) 6.25mg.ml^{-1}; (–▾–) 0.78mg.ml^{-1}; (–▽–) 0.39mg.ml^{-1}; (–▴–) 0.195mg.ml^{-1}; (–△–) 97.6µg.ml^{-1}; (–◇–) control. Points represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3).
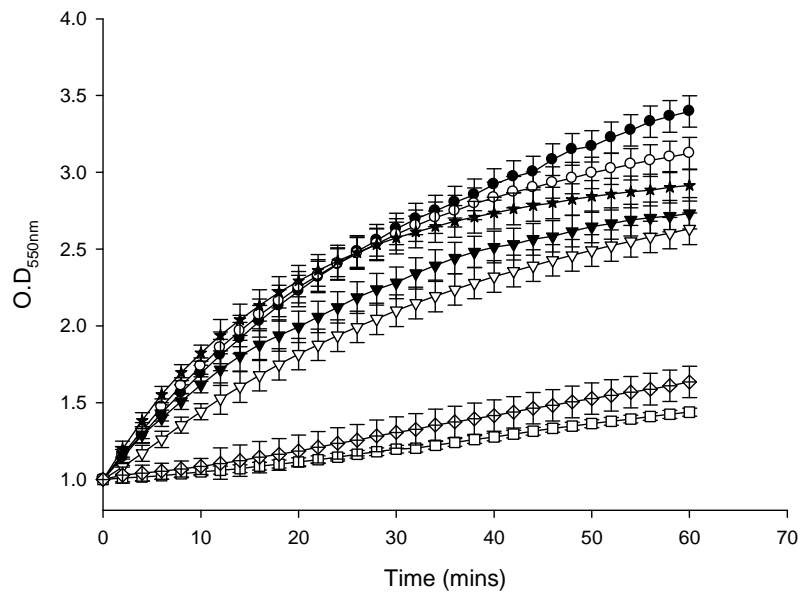
Figure 3.42: The effect of different concentrations of triclosan on INT activities of *S. oralis*; (–•–) 100µg.ml^{-1}; (–⊖–) 50.0µg.ml^{-1}; (–★–) 25.0µg.ml^{-1}; (–▼–) 12.5µg.ml^{-1}; (–▽–) 6.25µg.ml^{-1}; (–□–) DMSO 1%; (–◇–) control. Points represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3).
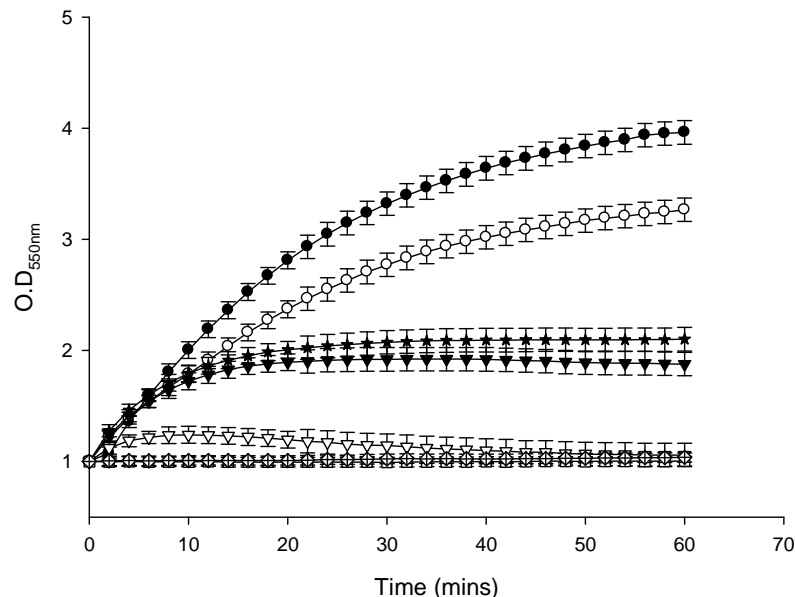
Figure 3.43: The effect of different concentrations of triclosan on TTC activities of *S. oralis*; (–•–) 100µg.ml^{-1}; (–⊖–) 50.0µg.ml^{-1}; (–★–) 25.0µg.ml^{-1}; (–▼–) 12.5µg.ml^{-1}; (–▽–) 6.25µg.ml^{-1}; (–□–) DMSO 1%; (–◇–) control. Points represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3).
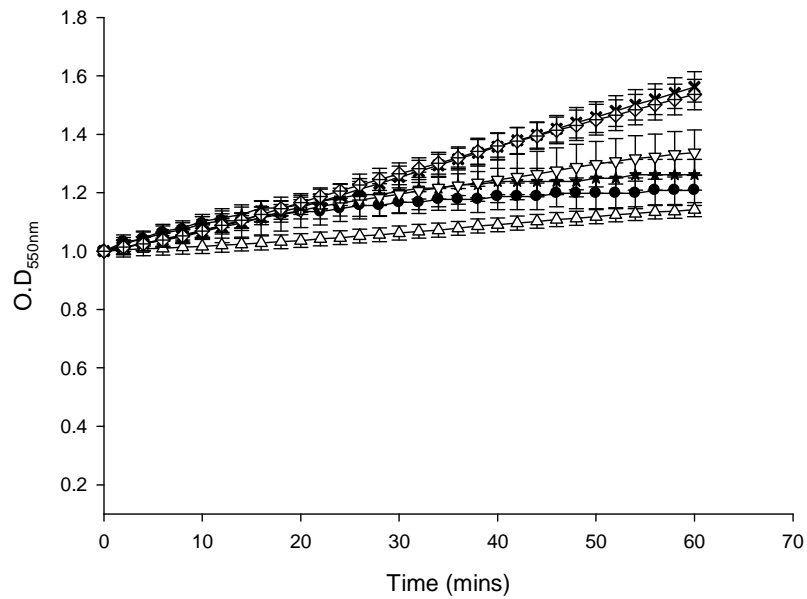
Figure 3.44: The effect of different concentrations of stannous fluordie on INT activities of *S. oralis*; (–●–) 1.25mg.ml^{-1}; (–★–) 0.31mg.ml^{-1}; (–▽–) 78.0µg.ml^{-1}; (–△–) 19.5µg.ml^{-1}; (–✕–) 2.43µg.ml^{-1}; (–◇–) control. Points represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3).
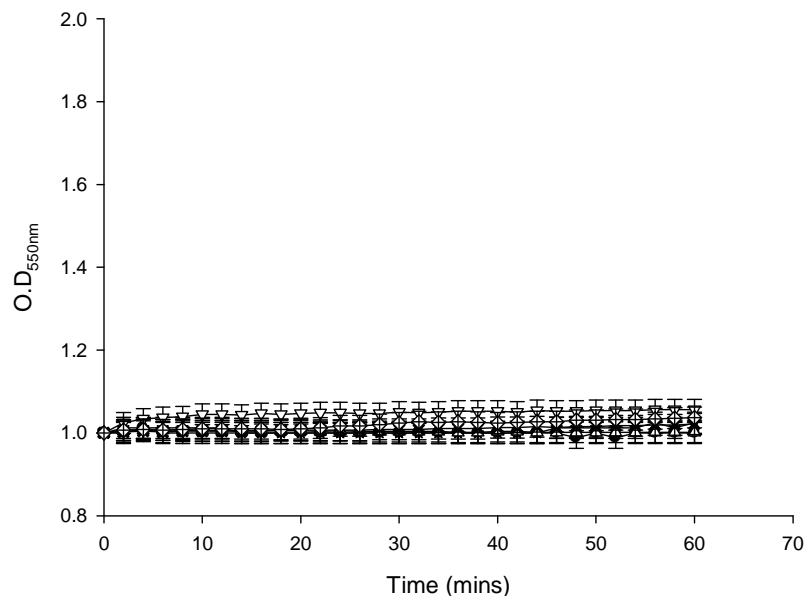
Figure 3.45: The effect of different concentrations of stannous fluordie on TTC activities of *S. oralis*; (–●–) 1.25mg.ml^{-1}; (–★–) 0.31mg.ml^{-1}; (–▽–) 78.0µg.ml^{-1}; (–△–) 19.5µg.ml^{-1}; (–✕–) 2.43µg.ml^{-1}; (–◇–) control. Points represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3).

The higher optical densities in the presence of INT dye produced by control cells could be explained by the metabolic pathway that the bacterial cells undergo. The metabolic pathways for control cells, in the absence of antimicrobial agents, are more likely to be of a glycolytic fermentative nature rather than complete oxidation of glucose. This metabolic behaviour of the control cells is explained by the higher affinity of INT dye to be reduced in the fermentation compared to the reduction of TTC. Low optical densities produced by TTC reduction in the presence of control cells could also explain a complete shut down of oxidative phosphorylation pathway in the control microorganisms. Data in Figs. 3.38 and 3.39 also show that glucose addition caused an increase in the optical densities which resembled a burst in the reduction of tetrazolium salts by *S. oralis* cultures in the presence of certain concentration of SLS antimicrobial. The increased reduction of tetrazolium salts in the presence of certain concentrations of antimicrobial agents could be caused by the shift of balance of use of carbon source from growth (anabolism) to complete oxidation (catabolism), i.e impaired or damaged cells need to conduct respiration to survive an injury rather than they need to grow (Baker *et al.* 1940; Gottschalk 1986). This high reduction behaviour was also noted in Figs. 3.42 and 3.43 in the presence of various concentrations of TCS, where a significant reduction of both tetrazolium salts was shown.

Data in Figs. 3.40 and 3.41 show that the optical densities were much higher in the presence of INT compared to that in the presence of TTC at certain concentrations of zinc lactate. This was the same case in the data presented in Figs. 3.44 and 3.45 in the presence of stannous fluoride, where the optical densities resembling INT reduction was higher than those of TTC.

Data in Figs. 3.46 and 3.48 show that the percentage reduction rate of TTC is higher than that of INT in the presence of various concentrations of SLS and triclosan. This data adds conformation to the data presented in Section 3.5.2.1.1 with cultures of *N. subflava* with the same antimicrobials. In which agents such as SLS and TCS have the ability to uncouple the oxidation of coenzymes such as NADH from the phosphorylation of ADP to ATP in the proton motive force. This effect results from the ability of these compounds to cause damage to the cellular membrane and make it more permeable to external protons resulting in short circuiting the ETS chain and the inhibition of ATP synthesis. Consequently, the cells need to respire at a faster rate to survive the injury produced by the uncoupling agents.
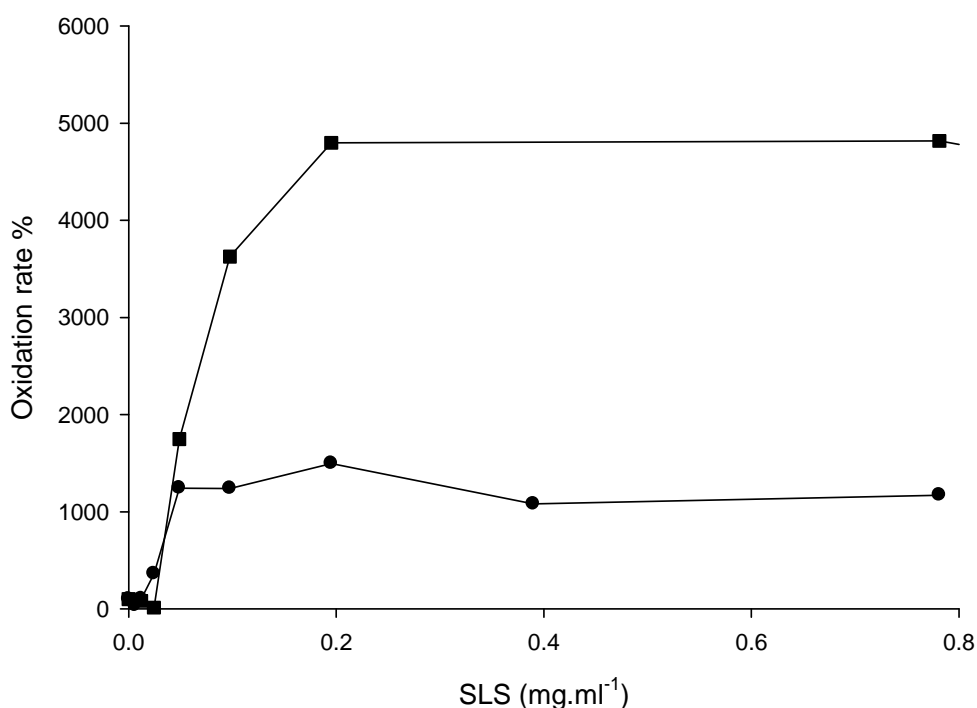
Figure 3.46: The percentage reduction rate of (—■—) TTC and (—●—) INT with *S. oralis* in the presence of sodium lauryl sulphate.
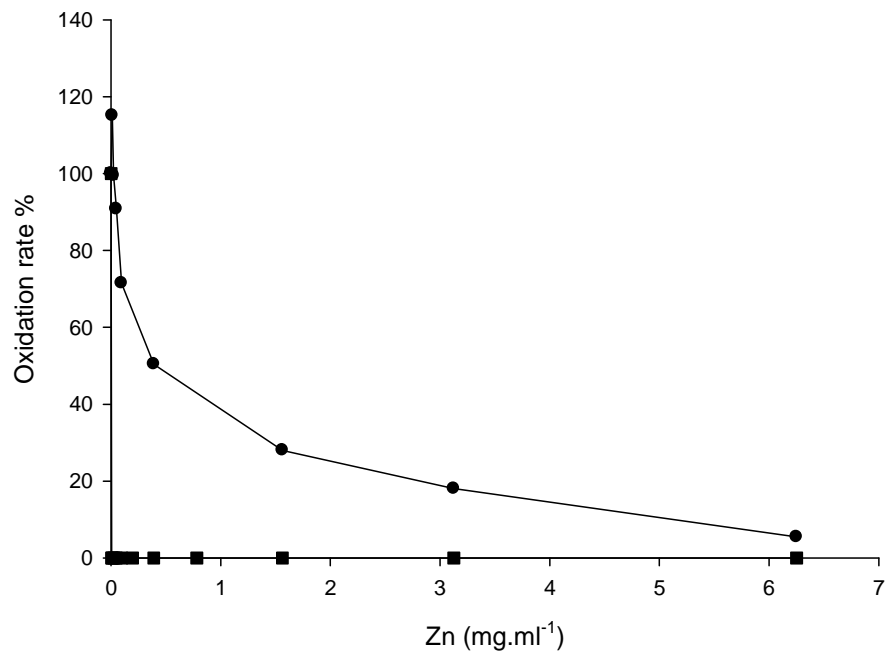
Figure 3.47: The percentage reduction rate of (—■—) TTC and (—●—) INT with *S. oralis* in the presence of zinc lactate.
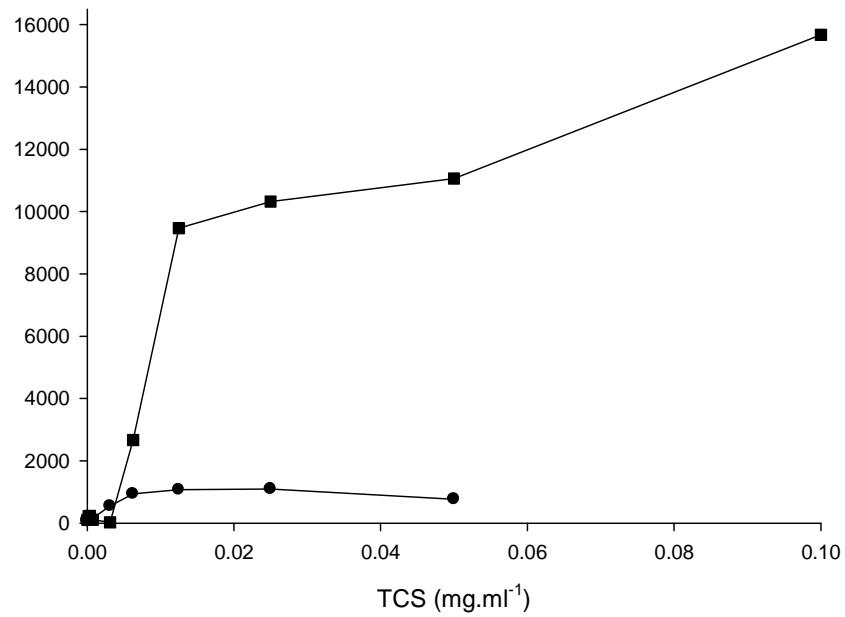
Figure 3.48: The percentage reduction rate of (—■—) TTC and (—●—) INT with *S. oralis* in the presence of triclosan.
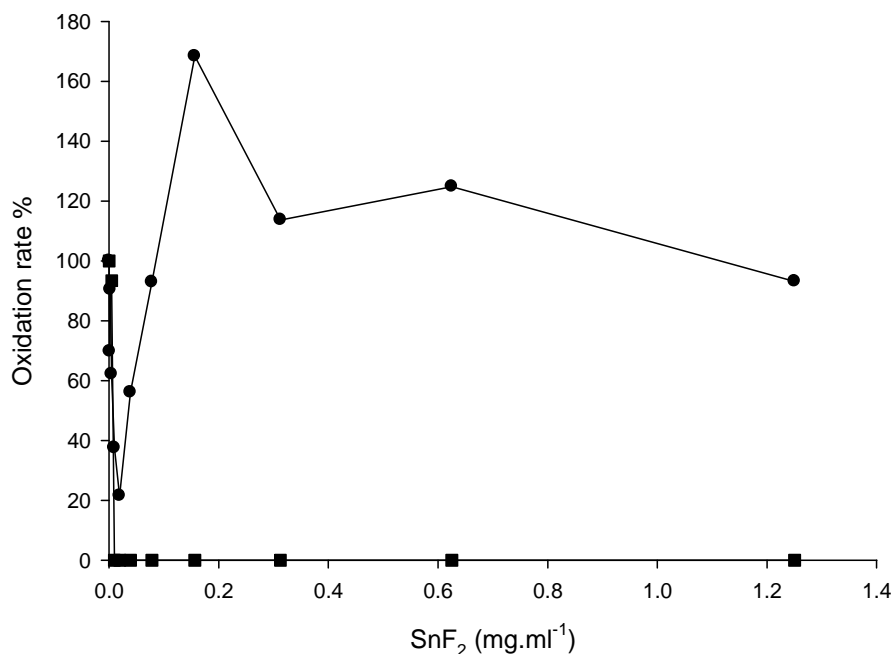
Figure 3.49: The percentage reduction rate of (–■–) TTC and (–●–) INT with *S. oralis* in the presence of stannous fluoride.

Higher percentage reduction rates of INT are presented in Figs. 3.47 and 3.49 in the presence of various concentrations of zinc lactate and stannous fluoride respectively. The same graphs also show inhibition in the reduction of TTC which is a clear indication that glucose oxidation was incomplete and the preferable metabolic pathway was fermentation rather than complete oxidation in the presence of sub-lethal levels of the correspondence antimicrobials.

3.6.2.1.3 Assessment of *E. coli* respiration activity

The effect of different concentrations of SLS on reduction potentials of INT and TTC dyes in *E. coli* are shown in Figs. 3.50 and 3.51. The optical densities representing the reduction potentials of INT and TTC were increased in the presence of certain concentrations of SLS (Figs. 3.50 and 3.51).
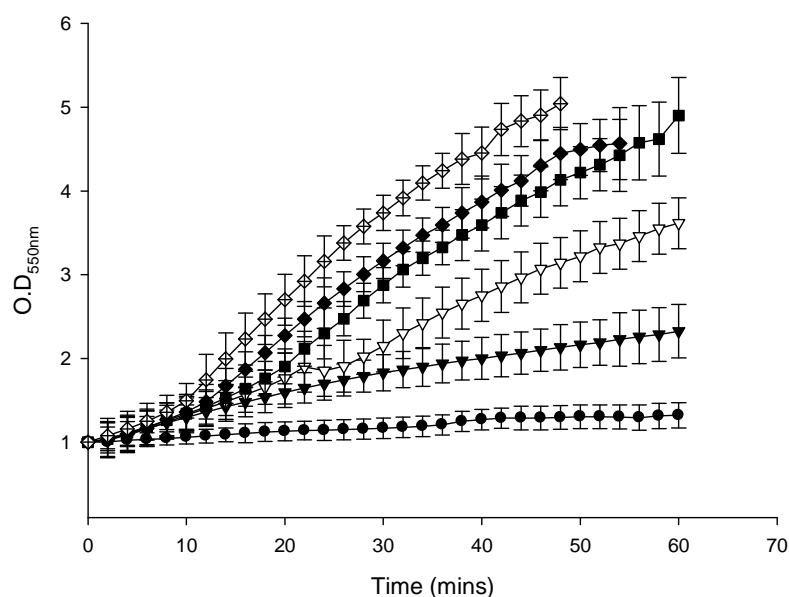
Figure 3.50: The effect of different concentrations of sodium lauryl sulphate on INT activities in *E. coli*; (—●—) 6.25mg.ml^{-1}; (—▼—) 0.78mg.ml^{-1}; (—▽—) 0.39mg.ml^{-1}; (—■—) 48.8µg.ml^{-1}; (—♦—) 24.4µg.ml^{-1}; (—◇—) control. Points represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3).
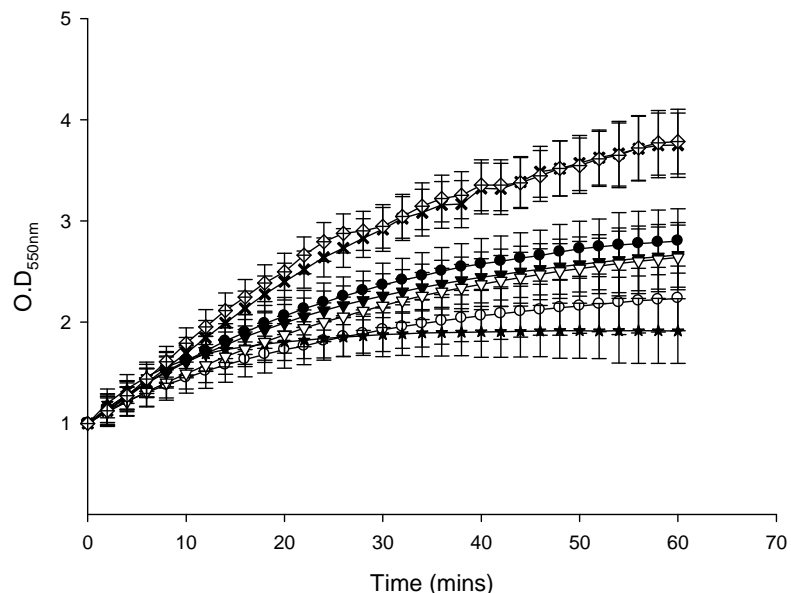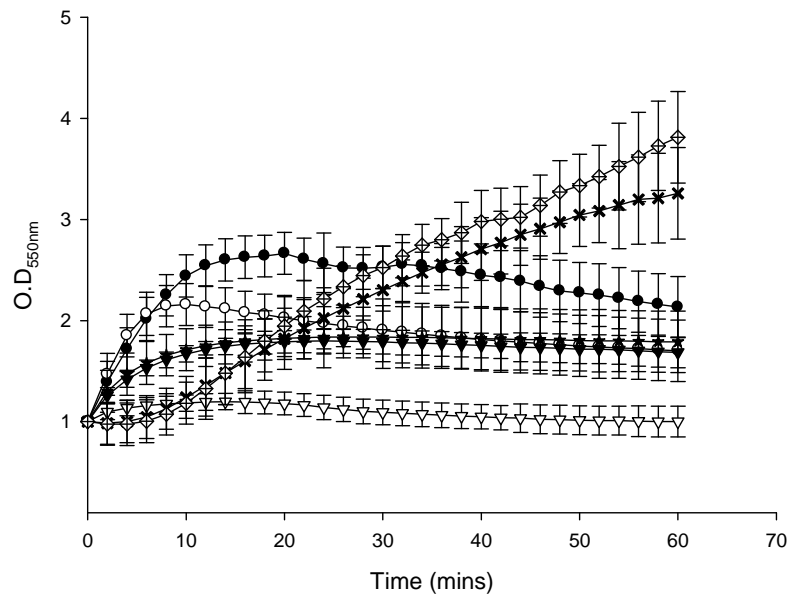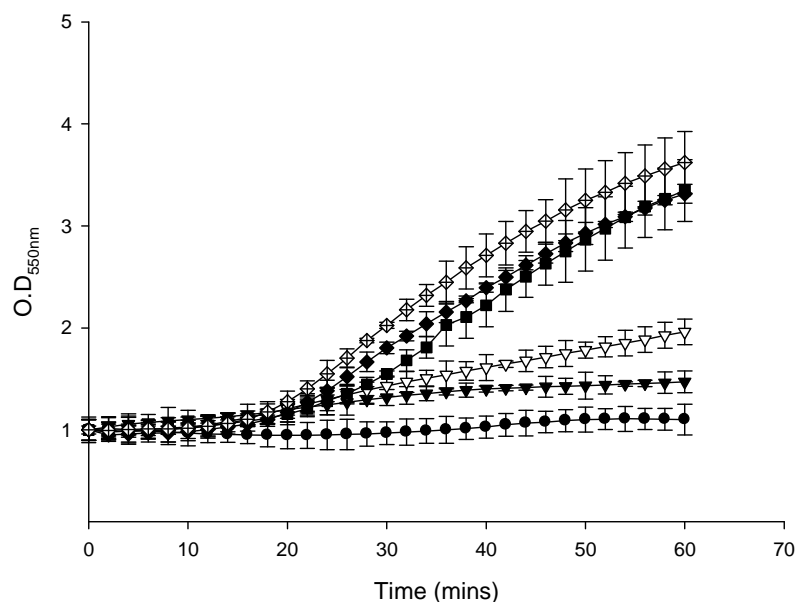

Figure 3.51: The effect of different concentrations of sodium lauryl sulphate on TTC activities in *E. coli*; (—●—) 6.25mg.ml^{-1}; (—▼—) 0.78mg.ml^{-1}; (—▽—) 0.39mg.ml^{-1}; (—■—) 48.8µg.ml^{-1}; (—♦—) 24.4µg.ml^{-1}; (—◇—) control. Points represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3).

122

These results were further investigated by calculating the percentage reduction rate of both dyes. In Fig. 3.52, plots of the calculated percentages of the reduction of INT and TTC show a similar rate of reduction in the presence of various concentrations of SLS, suggesting an equal reduction of both dyes. The respiratory behaviour of *E. coli* culture is more likely to be a complete oxidation of glucose in the ETS chain in the cytoplasmic membrane.
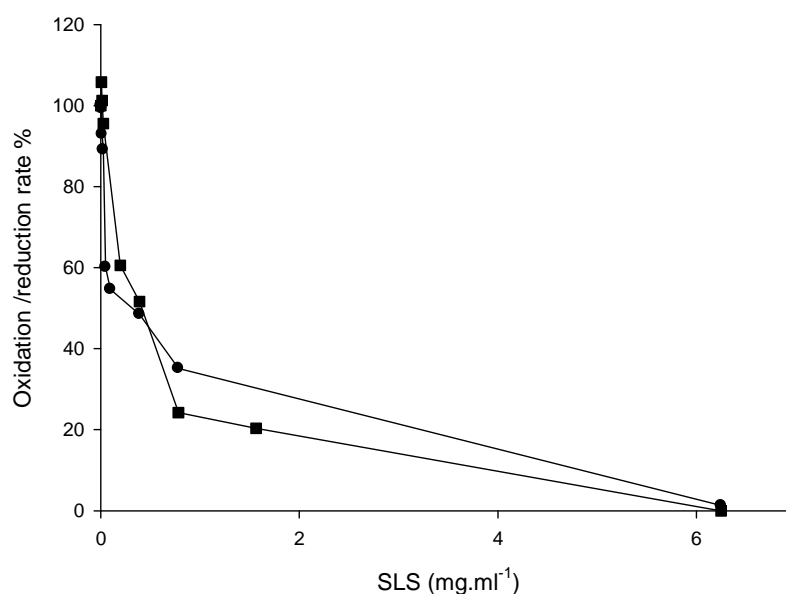
Figure 3.52: The percentage reduction rate of (–■–) TTC and (–●–) INT with *E. coli* in the presence of sodium lauryl sulphate.

Both Figs. 3.53 and 3.54 show an increased optical density in the presence of various concentrations of zinc lactate. Although, INT and TTC were reduced in the presence of zinc lactate, calculations of percentage reduction rate in Fig. 3.55 show a slightly greater reduction in favour of INT rather than TTC. High optical densities resembling reduction of the dyes were indicated at the onset of addition glucose/dye solution in the presence of TCS as an antimicrobial (Figs. 3.55 and 3.57).
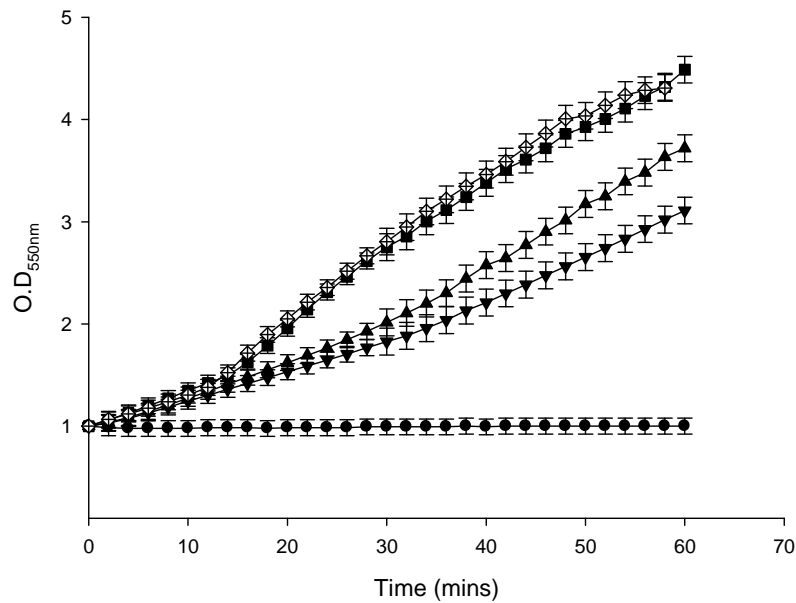
Figure 3.53: The effect of different concentrations of zinc lactate on INT activities in *E. coli*; (—●—) 6.25mg.ml^{-1}; (—▼—) 0.78mg.ml^{-1}; (—▲—) 0.195mg.ml^{-1}; (—■—) 48.8µg.ml^{-1}; (—◇—) control. Points represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3).
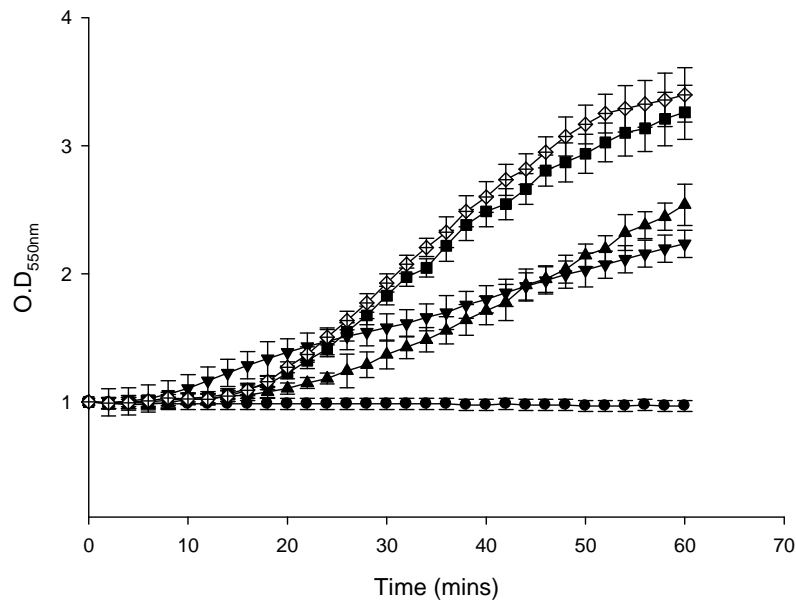

Figure 3.54: The effect of different concentrations of zinc lactate on TTC activities in *E. coli*; (—●—) 6.25mg.ml^{-1}; (—▼—) 0.78mg.ml^{-1}; (—▲—) 0.195mg.ml^{-1}; (—■—) 48.8µg.ml^{-1}; (—◇—) control. Points represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3).

This burst of the respiratory behaviour was thought to be an uncoupling effect in the presence of high concentrations of triclosan as previously described (Sections 3.6.2.1.1 and 3.6.2.1.2).
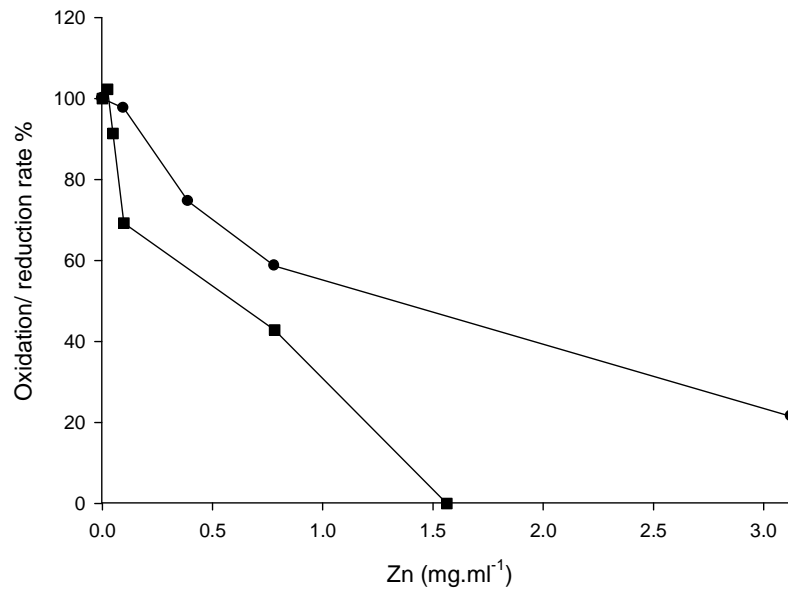
Figure 3.55: The percentage reduction rate of (–■–) TTC and (–●–) INT with *E. coli* in the presence of zinc lactate.
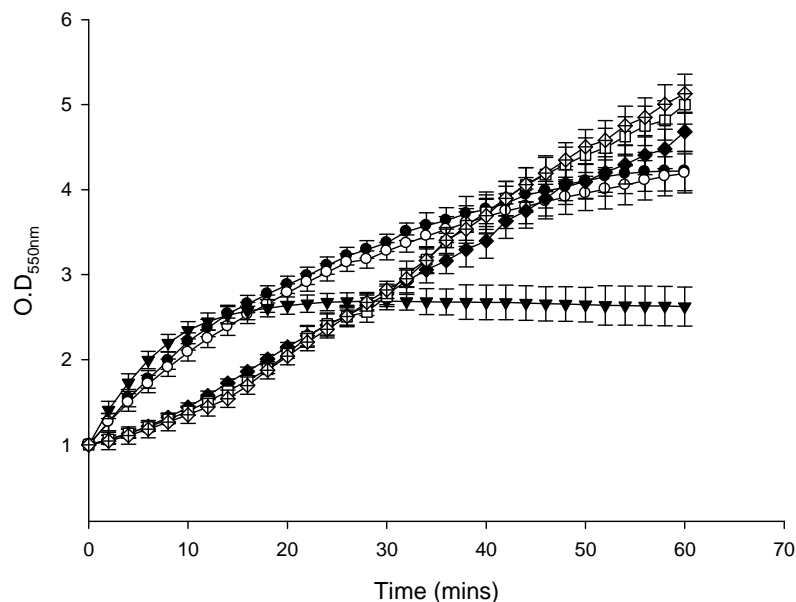
Figure 3.56: The effect of different concentrations of triclosan on INT activities in *E. coli*; (–●–) 100µg.ml^{-1}; (–○–) 50.0µg.ml^{-1}; (–▼–) 12.5µg.ml^{-1}; (–◆–) 0.4µg.ml^{-1}; (–□–) DMSO 1%; (–◇–) control. Points represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3).

The calculated percentage reduction rate presented in Fig. 3.58 shows an increased level of reduction percentage rate at high concentrations of TCS especially with TTC as an artificial electron acceptor.
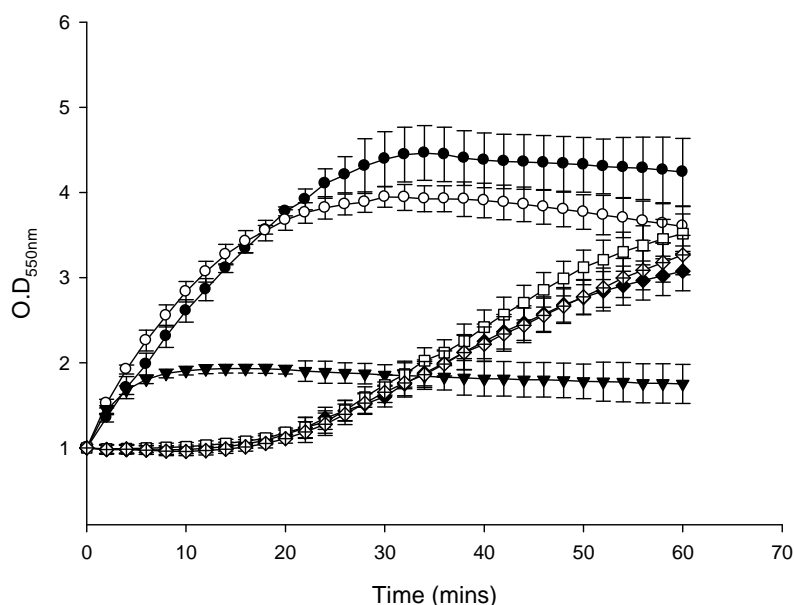
Figure 3.57: The effect of different concentrations of triclosan on TTC activities in *E. coli*; (–•–) 100µg.ml^{-1}; (–○–) 50.0µg.ml^{-1}; (–▼–) 12.5µg.ml^{-1}; (–◆–) 0.4µg.ml^{-1}; (–□–) DMSO 1%; (–◇–) control. Points represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3).
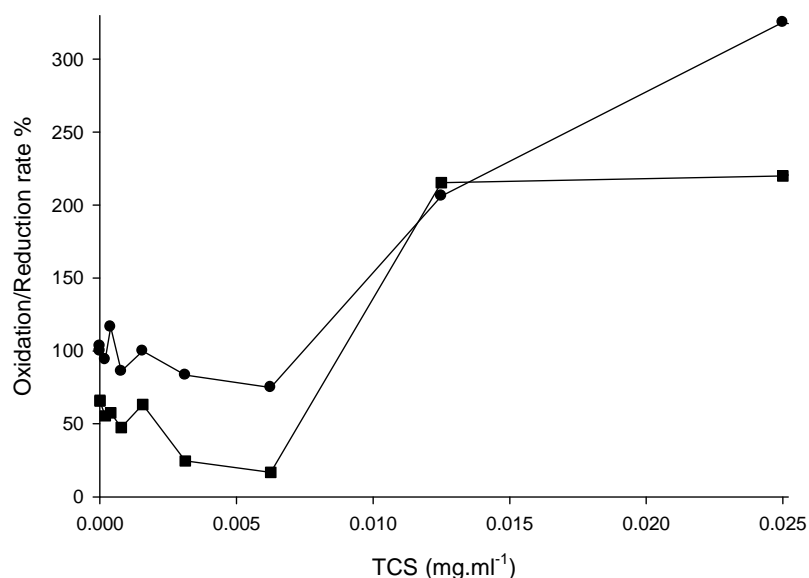
Figure 3.58: The percentage reduction rate of (–■–) TTC and (–•–) INT with *E. coli* in the presence of triclosan.

The reduction of INT dye with various concentrations of stannous fluoride was higher than that of TTC as indicated by the increased optical densities of these dyes in Figs. 3.59 and 3.60.
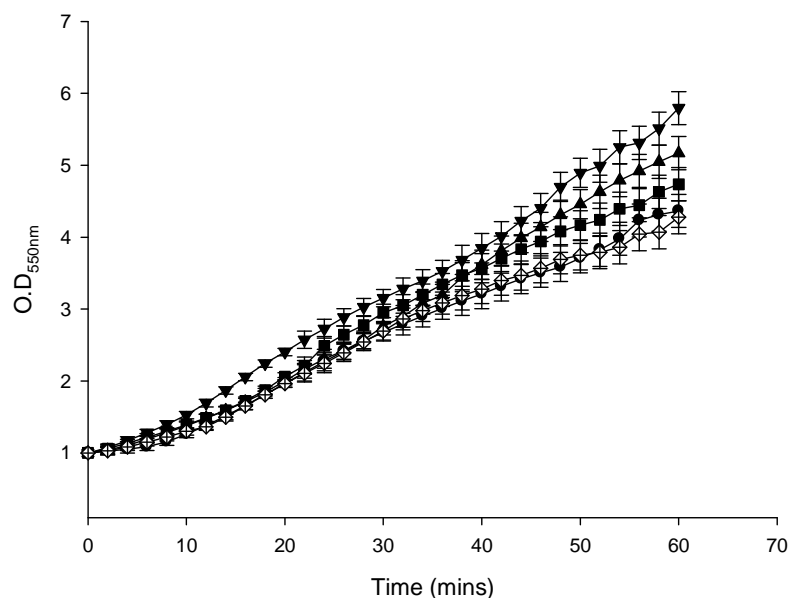
Figure 3.59: The effect of different concentrations of stannous fluoride on INT activities in *E. coli*; (−●−) 1.25mg.ml^{-1}; (−▼−) 156µg.ml^{-1}; (−▲−) 39.0µg.ml^{-1}; (−■−) 9.75µg.ml^{-1}; (−◇−) control. Points represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3).
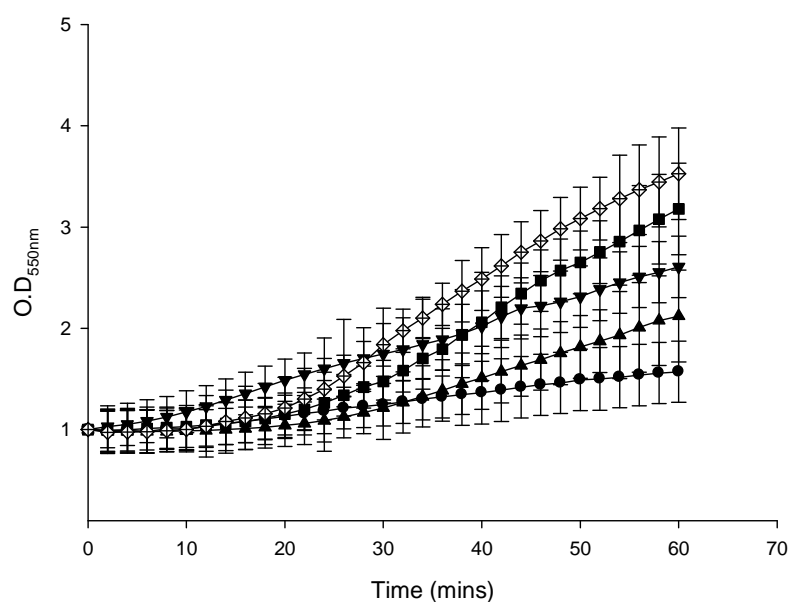
Figure 3.60: The effect of different concentrations of stannous fluoride on TTC activities in *E. coli*; (−●−) 1.25mg.ml^{-1}; (−▼−) 156µg.ml^{-1}; (−▲−) 39.0µg.ml^{-1}; (−■−) 9.75µg.ml^{-1}; (−◇−) control. Points represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3).

These observations were further confirmed by calculating and comparing the percentage reduction rates of both dyes (Fig. 3.61), which show a higher percentage reduction rate of INT compared to TTC in the presence of the same concentrations of zinc lactate. These results indicate that a glycolytic fermentative pathway is more likely to be the metabolic pathway conducted by the tested microorganisms in the presence of zinc lactate.
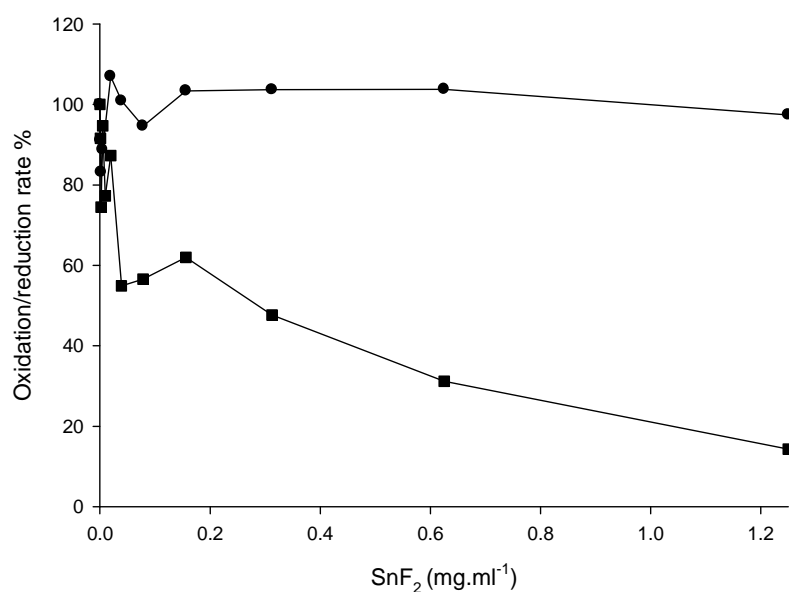
Figure 3.61: The percentage reduction rate of (–■–) TTC and (–●–) INT with *E. coli* in the presence of stannous fluoride.

In summary, damaged or impaired bacterial cells showed a higher respiratory activity in order to survive injury, rather than growing, compared to the control cultures. This observation was shown in all the bacterial cultures (*N. subflava*, *S. oralis* and *E. coli*) tested in the presence of certain concentrations of sodium lauryl sulphate and triclosan.

An example of such behaviour was the uncoupling of cellular respiration of all three bacterial cultures tested in the presence of various concentrations of SLS and TCS separately, as shown by the higher reduction of the tetrazolium dye TTC.

In addition facultative anaerobes (*E. coli* and *S. oralis*) showed a greater tendency to undergo fermentative metabolic pathways rather than complete oxidation of the substrate glucose in the presence of zinc lactate and stannous fluoride, as detected by the higher reduction of INT compared to TTC.

3.6.2.2 Respiratory activity detection with various substrates (short-term antimicrobial exposure)

The growth of bacterial cultures using different substrates as carbon sources was conducted in order to monitor the metabolic pathways targeted by sub-lethal concentrations of various oral hygienic molecules by the method described in Section 2.9.

A change in substrate for growth necessitates a change in the metabolic pathway and enzymes equipped for its utilization and oxidation. Therefore, adding a specific substrate that is involved in the cellular respiratory pathway enables the investigation and detection of the pathway that takes place in respiration in the presence of potential reduction dyes and the tested antimicrobial agent.

The growth on acetate as a carbon source was first studied by Sir Hans L. Kornberg in the late 1950s. According to Kornberg, acetate is transported into the bacterial cell and converted into acetyl-Co A by the action of acetyl-Co A synthetase and then Acetyl-Co A is fed into the TCA cycle, thus yielding NADH (Kornberg 1965). NADH-dependant dehydrogenases can be freely oxidized by INT for the detection of the TCA pathway. Therefore, the growth of microorganisms on acetate promotes the detection of respiratory pathways involved with oxidative phosphorylation rather than glycolysis and fermentation, i.e; acetate misses the

glycolytic and fermentation pathway when oxidized as the sole carbon source for bacteria.

A problem found in microorganisms while growing on acetate is how intermediates of the TCA cycle such as oxaloacetate are regenerated to serve as starting materials for cellular biosynthesis. In *E. coli*, the cleavage of isocitrate is catalysed by isocitrate lyase to form succinate and glyoxylate intermediates. Glyoxylate is then condensed with acetyl-Co A by the action of malate synthase to yield L-malate which serve as a precursor for oxaloactate. These extra reactions together with the TCA cycle reactions form the glyoxylate cycle (Gottschalk 1986).

The growth of bacteria on pyruvate as a carbon source was also employed in this study. Pyruvate oxidation was utilized to investigate the cellular metabolic pathways at sites of reduction of INT and TTC in both the fermentation and oxidative phosphorylation pathways. i.e; pyruvate, like acetate, misses the glycolytic pathway but it is still liable for the fermentative pathway or oxidative phosphorylation. The pathway of pyruvate oxidation is simple. Under anaerobic conditions pyruvate is converted into its fermentative products, lactate or alcohol, whilst under sufficient oxygen environment, pyruvate is transported into the cell and is oxidised by pyruvate dehydrogenase complex to acetyl-Co A which is then fed into the TCA cycle. Thus, the glycolytic pathway cannot be investigated when bacterial growth is mainly dependant on pyruvate.

The metabolic activity (INT and TTC reduction) of each bacterial species was observed spectrophotometricaly in the presence of each substrate and the optical densities were plotted as a function of time. The linear regression from each reduction curve was used to calculate the rate of reduction as a percentage of the control slope and plotted against the antimicrobial concentration.

3.6.2.2.1 Effect of antimicrobial exposure on respiratory rates detection in *N. subflava*

Data presented in Fig. 3.62 show a higher increase in the percentage of reduction rate of TTC than that of INT in the presence of different substrates as carbon sources and sub-lethal concentrations of SLS.
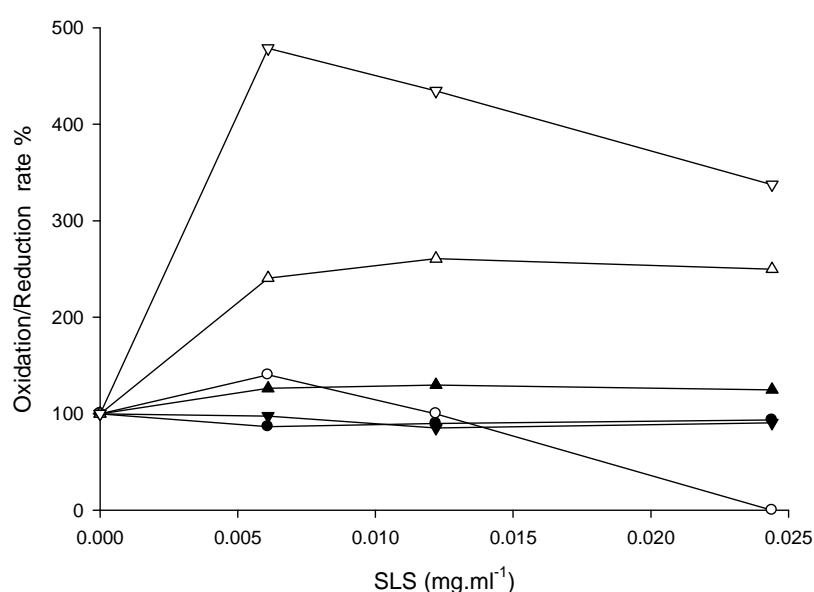
Figure 3.62: The percentage reduction rate of TTC & INT with *N. subflava* in the presence of different substrates; (–•–) INT+acetate; (–○–) TTC+acetate; (–▲–) INT+glucose; (–△–) TTC+glucose;(–▼–) INT+pyruvate; (–▽–) TTC+pyruvate, and sodium lauryl sulphate as an antimicrobial.

The higher reduction rate of TTC with all substrates in the presence of sub-lethal levels of SLS indicates the following; *i)* Growth on acetate inhibits microorganisms from undergoing glycolysis or fermentation. TTCs are highly reduced at the level of electron transport chain specifically cytochrome c oxidase in the cytoplasmic membrane. Higher reduction rates indicate that the metabolic pathway is a complete oxidation of the substrate in the ETS (Fig. 3.62). *ii)* Using glucose as a carbon source enables the microorganism to utilize both the glycolytic fermentative

pathway and oxidative pathways. Higher rates of reduction of TTC reduce the possibilities of a glycolytic/fermentative pathway (Fig. 3.62). *iii)* When pyruvate was employed as a substrate, it was expected that bacterial metabolic pathways would exclude glycolysis; TTC higher reduction rates confirm this hypothesis, showing a much greater probability of undergoing complete oxidation of the substrate (Fig. 3.62). *iv)* Although the exposure time to the antimicrobial was for 1 hr, the boost in the respiratory activity was marked. This activity indicated an uncoupling effect caused by SLS which was also detected in the presence of sub-lethal levels of triclosan, especially in the presence of glucose as a substrate (Fig. 3.64). The data presented in Fig. 3.62 also clearly shows that the respiratory behaviour of *N. subflava* cultures were complete oxidation of substrates in the presence of sub-lethal levels of SLS rather than fermentative pathway. This pattern of reduction was different in the presence of sub-lethal levels of zinc lactate (Fig. 3.63). The data presented in Figs. 3.63 and 3.65 show a higher reduction rate of INT than that of TTC in the presence of acetate as a substrate. Although INT reduction rate was higher, this does not necessarily mean that bacterial respiration is not undergoing complete oxidation. This may be due to either the ability of INT to be reduced in the ETS similarly to TTC, or that the substrate employed in the experiment (acetate) only allowed bacteria to commence oxidative respiration.

The metabolic activity of *Neisseria* cultures in the presence of glucose and zinc or stannous fluoride (Figs. 3.63 and 3.65) confirm the results presented with acetate in which oxidation is more likely to be the pathway of choice for this species under the specified conditions.
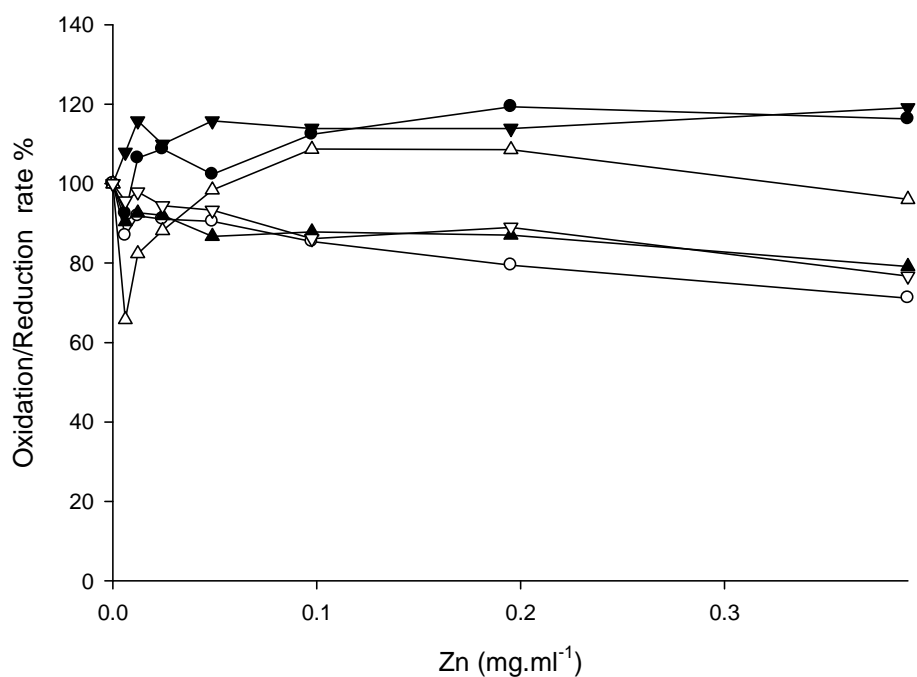
Figure 3.63: The percentage reduction rate of TTC & INT with *N. subflava* in the presence of different substrates; (–•–) INT+acetate; (–○–) TTC+acetate; (–▲–) INT+glucose; (–△–) TTC+glucose;(–▼–) INT+pyruvate; (–▽–) TTC+pyruvate, and zinc lactate as an antimicrobial.

When pyruvate was introduced as a carbon source, a similar respiration pattern to that with acetate was observed (Fig. 3.62). This similarity in reduction pattern could also explain a similarity in the respiratory activity of the bacterial cultures.
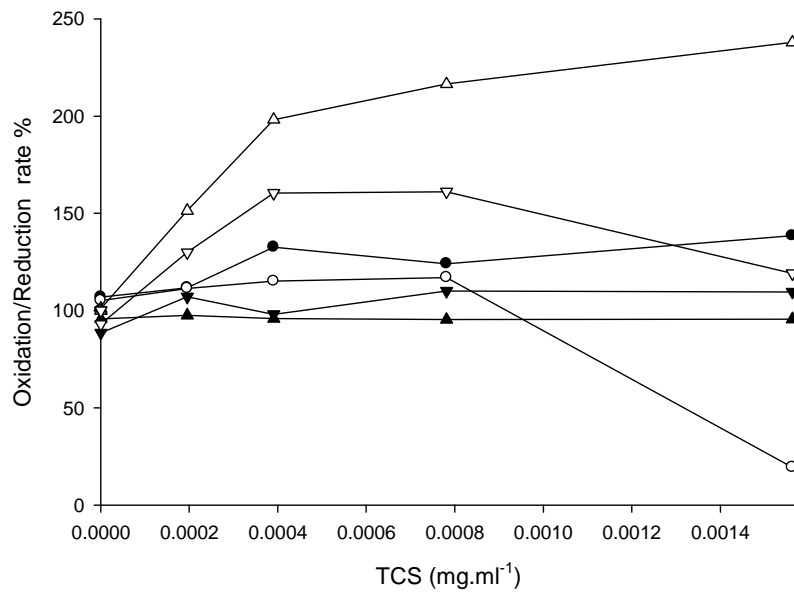
Figure 3.64: The percentage reduction rate of TTC & INT with *N. subflava* in the presence of different substrates; (–•–) INT+acetate; (–○–) TTC+acetate; (–▲–) INT+glucose; (–△–) TTC+glucose;(–▼–) INT+pyruvate; (–▽–) TTC+pyruvate, and triclosan as an antimicrobial.
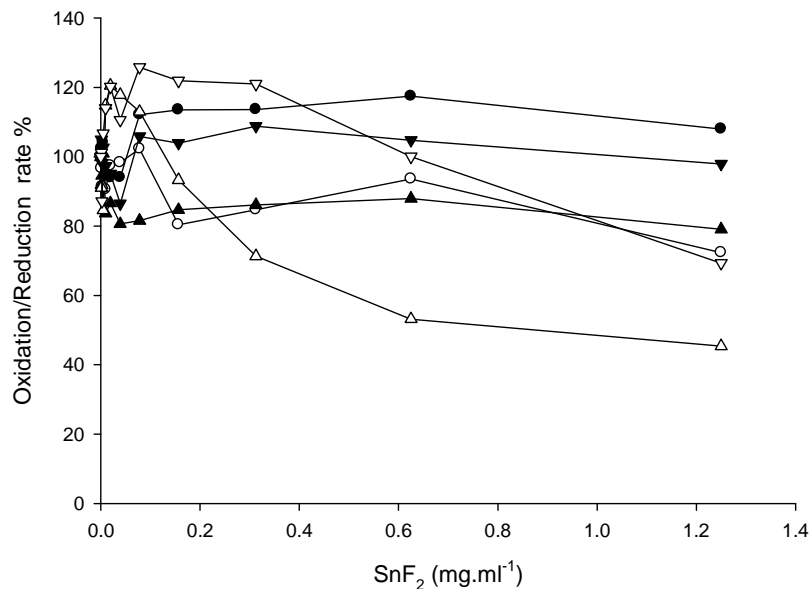
Figure 3.65: The percentage reduction rate of TTC & INT with *N. subflava* in the presence of different substrates; (–•–) INT+acetate; (–○–) TTC+acetate; (–▲–) INT+glucose; (–△–) TTC+glucose;(–▼–) INT+pyruvate; (–▽–) TTC+pyruvate, and stannous fluoride as an antimicrobial.

3.6.2.2.2 Effect of antimicrobial exposure on respiratory rates detection in *S. oralis*

The high reduction rates of TTC show an increased stimulation in the metabolic activity of *S. oralis* in the presence of SLS (Fig. 3.66). It was clearly noted that the reduction of TTC was substantially higher than that of INT in the presence of all the tested substrates, suggesting that complete oxidative phosphorylation was the preferable respiratory pathway in the presence of various concentrations of SLS.
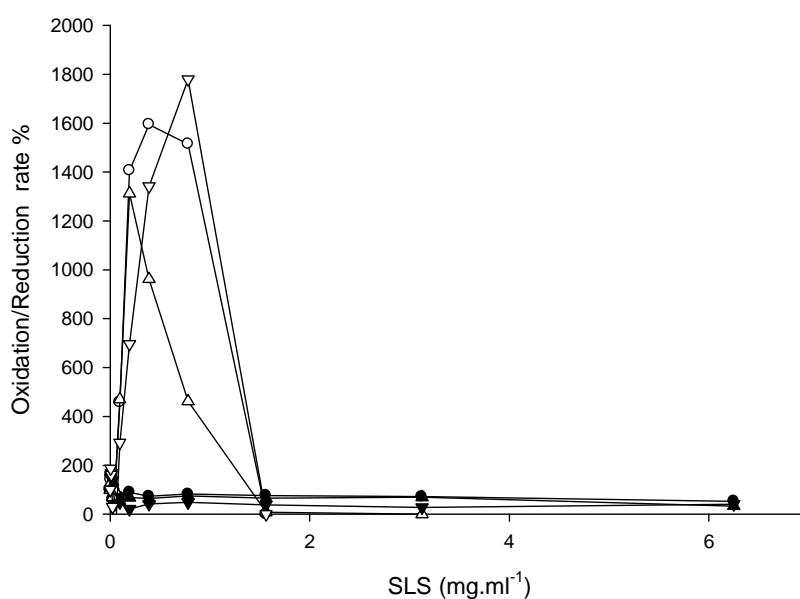
Figure 3.66: The percentage reduction rate of TTC & INT with *S. oralis* in the presence of different substrates; (–•–) INT+acetate; (–○–) TTC+acetate; (–▲–) INT+glucose; (–△–) TTC+glucose;(–▼–) INT+pyruvate; (–▽–) TTC+pyruvate, and sodium lauryl sulphate as an antimicrobial.

This stimulation in the metabolic activity was also shown in Fig. 3.67 where the respiratory activity of cells exposed to triclosan was much higher than in the control cells. This increase in the respiratory activity indicates an uncoupling effect on damaged cells caused by either SLS or TCS separately. Unlike with *N. subflava* cultures, this uncoupling effect with *S. oralis* cultures was observed at concentrations much higher than the sub-lethal levels of the antimicrobials. This could be a result of the short term exposure of microorganism to each antimicrobial.
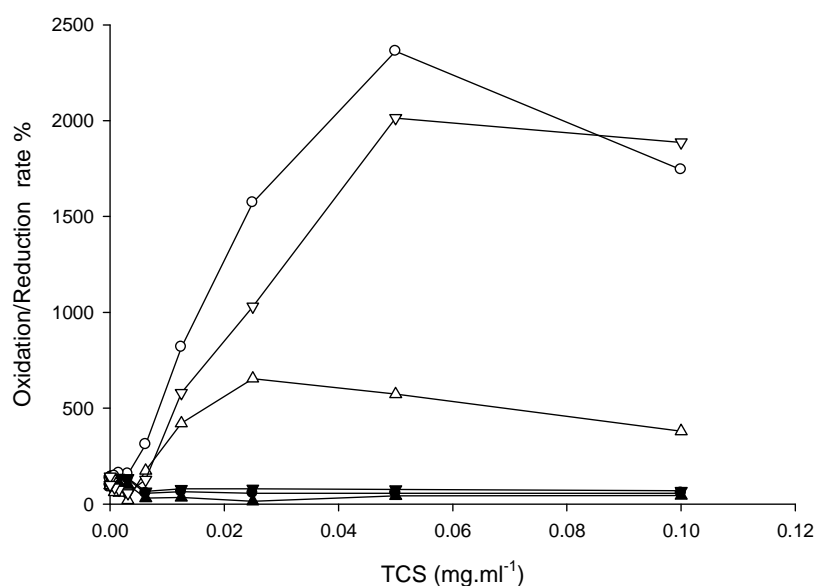
Figure 3.67: The percentage reduction rate of TTC & INT with *S. oralis* in the presence of different substrates; (–•–) INT+acetate; (–○–) TTC+acetate; (–▲–) INT+glucose; (–△–) TTC+glucose;(–▼–) INT+pyruvate; (–▽–) TTC+pyruvate, and triclosan as an antimicrobial.

Although fermentative pathways were observed in long-term exposure of *S. oralis* to stannous and zinc (Figs. 3.47 and 3.49), short-term exposure of *S. oralis* cultures to the same metals did not shift the metabolic pathways to the same extent (Figs. 3.68 and 3.69). This could be simply due to the short contact time. An increase in the reduction rate of tetrazolium salts occurred at concentrations up to 78µg.ml^{-1} for stannous fluoride (Fig. 3.68) and up to 100µg.ml^{-1} for zinc lactate (Fig. 3.69). This increase in the reduction rates in sub-lethal concentrations of antimicrobials could reflect the increase in the respiratory activity of damaged cells as noticed and explained previously (Section 3.5.2.1.1).
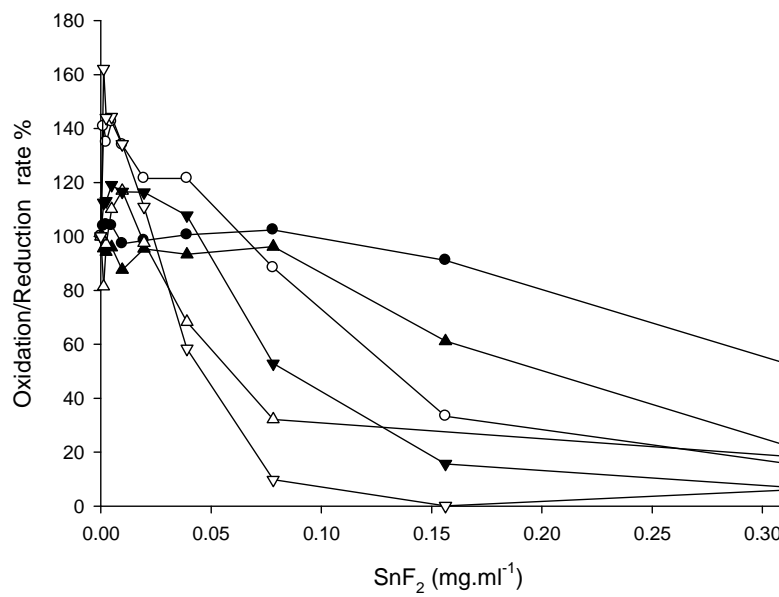
Figure 3.68: The percentage reduction rate of TTC & INT with *S. oralis* in the presence of different substrates; (–•–) INT+acetate; (–○–) TTC+acetate; (–▲–) INT+glucose; (–△–) TTC+glucose;(–▼–) INT+pyruvate; (–▽–) TTC+pyruvate, and stannous fluoride as an antimicrobial.
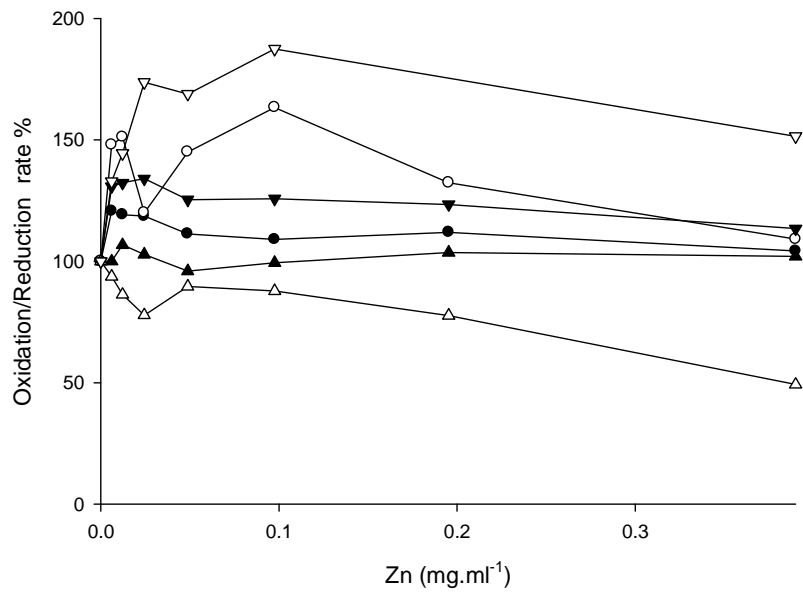
Figure 3.69: The percentage reduction rate of TTC & INT with *S. oralis* in the presence of different substrates; (–•–) INT+acetate; (–○–) TTC+acetate; (–▲–) INT+glucose; (–△–) TTC+glucose;(–▼–) INT+pyruvate; (–▽–) TTC+pyruvate, and zinc lactate as an antimicrobial.

In summary, the tested bacterial species showed a complete oxidative respiration pathway in the presence of all antimicrobial agents tested separately using different substrates. It is noted that detecting the respiratory pathways using different substrates has failed to detect any fermentative pathways, as previously shown in long-term exposure to various antimicrobial agents. The short time of exposure (1 hr) of different bacterial cultures to the sub-lethal levels of the tested antimicrobial agents might explain the inability of these agents to affect the metabolic pathways in the bacterial cells, therefore, it was more likely that tested cultures commence a complete oxidation pathway in the presence of sufficient oxygen rather than shifting to other pathways, hence the inability to detect fermentation. In spite of the failure to detect fermentation, uncoupling of bacterial respiration was confirmed and clearly indicated in the presence of SLS and TCS separately with different sugars.

3.7 Discussion

The control of plaque accumulation remains the main method for the prevention of dental plaque-related diseases such as periodontitis, gingivitis and dental caries. The maintenance of oral hygiene has proven to be enhanced when antimicrobial agents have been incorporated as adjuncts in dental hygiene formulations (Gordon *et al.* 1985; Svatun *et al.* 1987; Mandel 1988; Svatun *et al.* 1990). Hence, dentifrices and mouthwashes used in daily routine control of oral hygiene often include antimicrobial agents such as metal salts (stannous fluoride and zinc lactate), phenolic compounds (triclosan) and surfactants (sodium lauryl sulphate). At the cellular level, some antimicrobial agents often used in oral hygiene products exert their anti-metabolic activity at sub-lethal levels by affecting the bacterial

cellular membrane and its metabolic pathways (Marsh 1994). Hence, it is physiologically relevant to study the influence of sub-lethal levels of oral antimicrobials on metabolic pathways such as respiration and glycolysis/fermentation. Another reason behind measuring cellular respiration/fermentation was to detect the glycolytic/fermentative pathway since, acidic fermentation products have been proven to act as cariogenic factors. Therefore, this chapter studied the effects of both effective and sub-lethal concentrations of several antimicrobial agents commonly incorporated in dentifrices and mouthwashes on bacterial growth and respiration/fermentation pathways of axenic bacterial species.

The effects produced by effective oral hygiene molecules have been previously studied and reviewed (Maltz and Emilson 1982; Ritchie and Jones 1988; Giertsen 2003). There is however, a lack of understanding of the effects produced by oral antimicrobial agents on metabolic pathways at sub-lethal concentrations. Thus, methods in the present chapter were performed using tetrazolium salts as bacterial metabolic pathways indicator in the presence of sub-lethal concentrations of various oral antimicrobial agents. The use of tetrazolium salts as biological indicators for measuring cells viability via detecting their respiratory activity has been previously studied (Zimmermann *et al.* 1978; Fukui and Takii 1989; Roslev and King 1993; Smith and McFeters 1997; Bhupathiraju *et al.* 1999). However, they have not been applied to oral hygiene agents in the manner described in this chapter.

Previous studies into the antimicrobial effects of compounds such as tetrachlorosalicylanilide, trichlorocarbanilide and dinitrophenol have indicated the ability to of such compounds to behave as bacterial respiration uncoupling agents

(Hamilton 1968; Russell 1992). However, data from this chapter suggest that the antimicrobials triclosan and sodium lauryl sulphate were also able to uncouple NADH from the phosphorylation of ADP to ATP and therefore behaving as uncoupling agents. This uncoupling activity can be a result of either the loss of sub-cellular fractions and impaired function of the cellular membrane or the antimicrobial agents being highly lipophilic and crossing the cell membrane freely and acting as proton carriers (Russell 1992). In both cases, the mobility of external protons may result in short circuiting the ETS chain and resulting in the inhibition of ATP synthesis. In uncoupling, substrates are almost completely oxidised in the presence of an uncoupling agent, and cellular components are not synthesised (Gottschalk 1986). Such metabolic behaviour was observed in both long- and short-term studies by detecting the optical densities of the reduced tetrazolium dyes in the presence of the tested antimicrobial agent. Dyes with different redox potentials provided information of the respiratory levels depending on the reduction percentage of the treated cells compared to their controls. Theoretically, the uncoupling of respiration of microorganisms in the oral cavity could serve as a benefit to oral health by the routine use of oral hygiene products containing uncouplers such as triclosan and sodium lauryl sulphate. This results in the inability of bacterial cells to grow. However, such exposed cells would continue to respire and thus utilize available substrates present in the oral cavity. This activity could result in plaque disruption. An example of such uncoupling behaviour can be observed by the inability of the tested species such as *S. oralis* to grow in the presence of 100µg.ml^{-1} of TCS (Fig. 3.20), whereas the same species were completely capable of reducing the tetrazolium dye, hence respiring at the same concentration as shown in Fig. 3.57.

Data presented in this chapter also suggest that long-term exposure (18 hrs) of facultative anaerobic species (*S. oralis* and *E. coli*) to sub-MIC of stannous fluoride, can result in the shift of the metabolic activity to fermentative pathways (Figs. 3.49 and 3.61). In an oral ecosystem such shifts in the metabolic behaviour of the affected microorganisms could lead to an adverse impact on the oral hygiene by providing potentially increasing acidogens (Bradshaw and Marsh 1988; Marsh and Martin 1999). Data for short-term exposure of bacterial species to different oral antimicrobial agents using different substrates did not indicate shifs in the metabolic pathways towards a fermentative reaction, especially in the presence of antimicrobials that have previously shown such affect. This observation could be explained by the insufficient contact time between the microorganisms and the tested antimicrobial agent which did not allow shifts in cellular metabolism. The study of the metabolic activity in the current chapter suggests that perhaps other mechanisms of action are responsible for the antimicrobial efficacies of some antimicrobial agents especially over short contact periods with bacterial species and this will be studied within the following chapters.

CHAPTER 4

An *in vitro* comparative evaluation of the anti-plaque effects of proprietary mouthwashes

4.1 Introduction

Dental plaque is a taxonomically diverse sessile microbial ecosystem which may contain more than 500 different bacterial species (Paster *et al.* 2001). This diverse bacterial community is found on healthy enamel and other surfaces in the mouth, but it is also involved in the pathogenesis of two of the most prevalent diseases affecting developed countries: caries and periodontal diseases. Dental caries and periodontal disease are the main causes of tooth loss in man (Addy *et al.* 1992). Attempts to control plaque accumulation or prevent its harmful effects continue to be means for the prevention of dental plaque-related diseases. In general, the prevention of dental diseases is more effective when antimicrobial agents are used to protect against plaque accumulation. Thus, there is a considerable interest in the use of antimicrobial agents as adjuncts to physical removal methods such as brushing and flossing to prevent dental disease. Because they serve as a valuable complement to mechanical plaque removal (i.e. brushing and flossing), antimicrobials have been incorporated into many different oral health formulations such as toothpastes, mouthwashes, sprays and gels. Over the years, a number of essential oil preparations (including thymol, menthol and eucalyptol); bisbiguanides (e.g. chlorhexidine), quaternary ammonium compounds (e.g. cetylpyridinium chloride), oxidising agents (e.g. hydrogen peroxide) and fluorinated compounds (e.g. sodium fluoride) have been tested and developed in commercial formulations and have been previously studied and reviewed (Holbeche *et al.*

1975; Gordon *et al.* 1985; McBain *et al.* 2003). However, there is a lack in understanding of the mechanisms by which they control the plaque ecosystem. Therefore, this chapter comparatively evaluates the antibacterial efficacy of various commercial antimicrobial-containing mouthwashes using an *in vitro* biofilm model. Understanding the antibacterial efficacy of different commercial mouthwashes would be further enhanced by investigating the characteristics of the active components presented in them. This chapter, therefore, will consider the mechanism of action of the active components of the mouthwashes described in Table 4.1 before studying their effects on complex *in vitro* plaque models.

4.1.1 Chlorhexidine

Chlorhexidine (CHX) is active against a variety of Gram-positive, Gram-negative bacteria and some fungi (Gilbert and Moore 2005). Chlorhexidine has been incorporated in a variety of oral hygiene products such as mouthwashes (Periogard®) and toothpastes (Curasept®). It has also been introduced in surgical hand washes as an antiseptic. The mechanisms of action of CHX are similar to those of the quaternary ammonium compounds; the bisbiguanide groupings in CHX have affinity for anionic sites of the cell membrane (Chawner and Gilbert 1989a; Chawner and Gilbert 1989c). This interaction causes CHX to form bridges between pairs of adjacent phospholipid head groups of the cell membrane and to displace the associated stabilising divalent cations (Davies 1973) causing a reduction in cell membrane fluidity and affecting osmoregulation and consequently metabolic capability of the cell (Hugo and Longworth 1966). Severe interactions may occur at higher concentrations causing the cell membrane to adopt a liquid crystalline state, loss of structural integrity and leakage of cellular materials

(Longworth 1971; Chawner and Gilbert 1989a; Chawner and Gilbert 1989b; Chawner and Gilbert 1989c). Another mechanism of action of CHX has been identified by Hastings (2000), which is the ability of CHX, following entry to the cytoplasm to cause inhibition of proteolytic and glucosidic enzymes. In 1970, Löe and Schiott were the first to demonstrate the anti-plaque activity of CHX (Loe and Schiott 1970). By 1974, CHX activity had proven to be one of the most effective anti-plaque agents and thus for the prevention of oral diseases (Adams and Addy 1994). The major advantage of CHX over some other anti-plaque compounds is its cationic nature that enables it to bind to both hard surfaces (tooth) and soft surfaces (oral mucosa), to maintain substantivity, i.e. enabling it to reduce pellicle formation and helping it to act over a long period of time after its use (McBain *et al.* 2003). Many studies have demonstrated the efficiency of CHX in reducing oral bacterial viability (Hase *et al.* 1998), inhibiting plaque regrowth and preventing gingivitis (Loe and Schiott 1970). The main disadvantage of CHX is an unpleasant taste and in some cases associated staining of the teeth (Addy *et al.* 1985), which at times can be severe and requires professional cleaning (Hoyos *et al.* 1977). Chlorhexidine has relatively low mammalian toxicity and therefore can be safely used in oral hygiene products (Foulkes 1973).

4.1.2 Cetylpyridinium chloride

As a monocationic quaternary ammonium compound, the mode of action of cetylpyridinium chloride (CPC) is thought to involve perturbation of the lipid bilayer of both the cytoplasmic and the outer membrane in Gram-negative microorganisms. This action on the membrane leads to a generalised and progressive leakage of cellular components to the environment (Gilbert and Moore

2005). Cetylpyridinium chloride-containing mouthwashes have been marketed since the early 1940s. Data obtained from various clinical studies have shown a reproducible significant reduction of supragingival dental plaque with the use of mouthwashes containing 0.05% to 0.1% CPC (Holbeche *et al.* 1975; Rawlinson *et al.* 2008). With CPC formulations, staining also occurs but to a lesser extent than for CHX-containing mouthwashes. However, taste alteration and/or burning sensations have been frequently reported with alcohol-based, CPC-containing mouthwashes (Rawlinson *et al.* 2008). Cetylpyridinium chloride has also proven to have low mammalian toxicity (Arro and Salenstedt 1973).

4.1.3 Sodium Fluoride

Sodium fluoride (NaF) has been incorporated in a wide range of oral hygiene products such as mouthwashes and dentifrices. The cariostatic effects of NaF have been extensively studied and reviewed (Bibby *et al.* 1946; Horowitz 1971; Andres *et al.* 1974; Sudjalim *et al.* 2007), and it has been concluded that the effects associated with NaF appear to be carried out by the fluoride ion rather than the metal component as in the case with stannous fluoride (Maltz and Emilson 1982). The cariostatic activity of the fluoride ion is thought to be a result of the physiochemical properties of the teeth which involves the incorporation of fluoride ions into the hydroxyapatite structure of the enamel of the tooth forming fluoroapatite. This incorporation involves the replacement of the hydroxyl groups and the redeposition of less soluble fluoridated hydroxyapatite (de Leeuw 2004). Other studies suggest that fluoride ions also interfere with initial oral bacteria resulting in plaque formation, affecting bacterial metabolism and inhibiting acid production (Bibby and Van Kerstern 1940; Hamilton 1977; Skjörland *et al.* 1978).

4.1.4 Hydrogen Peroxide

The bactericidal action of H_2O_2 is related to its oxidising effect through the release of oxygen in the presence of tissue or bacterial-derived enzymes (Miyasaki *et al.* 1986). Therefore, it is not surprising that H_2O_2 has been used to control or suppress oral infections caused by obligate anaerobes, due to the high sensitivity of these species to active oxygen (Imlay 2002). Mouthwashes containing 3% H_2O_2 have shown great effectiveness in the reduction of VSC production (Suarez *et al.* 2000). The anti-plaque and anti-gingivitis effects of H_2O_2 containing mouthwashes have also been reported but remain in dispute due to contradictory findings and the limitation of information related to this agent (Wennstorm and Lindhe 1979; Gusberti *et al.* 1988). Hydrogen peroxide is used as an active component in a number of over-the-counter oral hygiene products such as (Peroxyl[®]) mouthwash.

4.1.5 Essential oils

A mixture of essential oils (thymol, menthol and eucalyptol) have been in use as a proprietary mouthwash Listerine[®] for over a century. A number of long-term clinical trials have examined the adjunctive benefit of this EO-containing mouthwash and its anti-plaque and anti-gingivitis efficacy (Gordon *et al.* 1985; DePaola and Daniel 1989; Daniel *et al.* 1990; Sharma *et al.* 2001; Sharma *et al.* 2004). Clinical studies have also suggested that brief exposure of microorganisms to an EO-containing mouthwash causes distinctive morphological changes in bacteria and the loss of cell-surface integrity, leading to an inhibition in enzymatic activity and cell death. This bactericidal action prevents secondary bacterial colonisers from aggregating with Gram-positive pioneer species, slowing down plaque formation, resulting in the reduction of bacterial load and decrease of

plaque mass (Kurbert *et al.* 1993; Fine *et al.* 2001). The capability of this type of mouthwash to penetrate the plaque biofilm and exert bactericidal action has been explained by Ouhayoun (2003). A short-term clinical study (two weeks) has also demonstrated the effectiveness of an EO-containing mouthwash in inhibiting the development of, and reducing existing plaque, in subjects who used no other oral hygiene procedures (Mankodi *et al.* 1987). In spite of its prolonged use as a mouthwash and its proven anti-gingivitis and anti-plaque activity, there is still a lack of understanding about the contribution of the active ingredient(s) that maybe responsible for its anti-plaque activity since most of the studies focused on the complete formulation rather than its individual components. Therefore, investigating the antimicrobial activity of the active agents an EO-containing (individually and in combination) was one of the aims of this current chapter.

4.2 Models/Approaches

For any *in vitro* model system to be a representation of real phenomena it must reproduce aspects of the ecosystem to be studied. Hence, the hydroxyapatite disc model (HDM), first described by Guggenheim *et al* (2001), consists of a disc made up of hydroxyapatite (HA) which is chemically similar to the tooth enamel. This model has the capability of harbouring microorganisms of complex ecosystems such as oral bacteria and can be used to represent a supragingival plaque system (Guggenheim *et al.* 2001). The current *in vitro* study was designed and executed in two parts; the first part was to use the HDM to demonstrate the ecological shifts in plaque composition (functional bacterial groups) in response to short-term exposure to oral chemoprophylactic agents (complete mouthwashes and/or active ingredients). The second part was to use a microscopic evaluation technique,

LIVE/DEAD bacterial-viability staining (BacLight; Molecular Probes, Leiden, The Netherlands), to evaluate the intact bacterial biomass on HA. The use of HA discs appeared to be an unsuccessful approach in the microscopic evaluation technique (data not presented) due to cross fluorescence between the bacterial biomass and the HA material, hence a glass slide model (GSM) was used to overcome the background cross fluorescence problem. Both the incubation periods and the methodology of the HDM and GSM were previously described in Sections 2.12 and 2.13.

4.3 Microorganism choice for validation studies of glass slide model

Although the use of glass surfaces as substrata has been described in several biofilm studies, their use for the growth and attachment of oral bacterial species individually and/or as whole salivary biofilms has not been validated (LeChevallier *et al.* 1988; Bowden and Li 1994; Shu *et al.* 1999; Mah and O'Toole 2001). It is acknowledged that plaque ecosystems are composed of several hundred bacterial species, each of which plays role in plaque architecture (Marsh and Martin 1999). Hence, the selection of microorganisms for this particular study was based on bacterial species comprising the early or primary colonisers of plaque biofilm. *S. oralis, S. sangius* and *N. subflava* were selected as a simple paradigm to be used in validation studies as the microorganisms resembling the early colonisers in the dental plaque (Marsh and Martin 1999). After successive validation experiments involving mono-species biofilms, the glass substrata were further validated by growing salivary microcosm biofilms on them as previously described (Section 2.13).

4.4 Aims

The antibacterial effects of various formulations used for the enhancement of oral hygiene is related to the active ingredient(s) as well as other components in the formulation (Marsh 1992). Here, the most potent oral formulations with the highest antibacterial efficacy were tested alongside their active component(s). Hence, the main aim of this chapter was to investigate the antimicrobial efficacy of different oral formulations and detect the ingredient(s) responsible for their bactericidal potency against functional groups of *in vitro* salivary microcosms.

4.5 Results

4.5.1 Statistical analysis

For analysis of viable counts, the Student's t-test was used to determine whether the use of glass surfaces for biofilm formation were significant for validation studies. The Student's t-test was also used to determine whether the effects of various mouthwashes in microcosms were significant. Data were arranged in tables and subjected to a SigmaPlot version 11 Systat[©] software.

4.5.2 Validation studies of glass slides models

Glass slides models have been tested comparatively with HDMs for their ability to be used as substrata for reproducing biofilms successfully with different oral bacterial species (pure bacterial cultures and whole salivary microcosms). This validation was considered to be important for further microscopic investigations of the effects of a proprietary mouthwash on biofilm attachment. Fig. 4.1 illustrates the ability *N. subflava* cultures to attach to different substrata (glass and HA) aerobically for 48 hrs. The total counts of *N. subflava* grown on each of the three

test glass surfaces were not significantly different to each other ($p > 0.05$). The total counts were also not significantly different ($p > 0.05$) when *N. subflava* was grown on each HA discs. Moreover, there was no significant difference in the viable counts of *N. subflava* grown on glass substrata compared to those grown on HA discs ($p > 0.05$) as shown in Fig. 4.1.
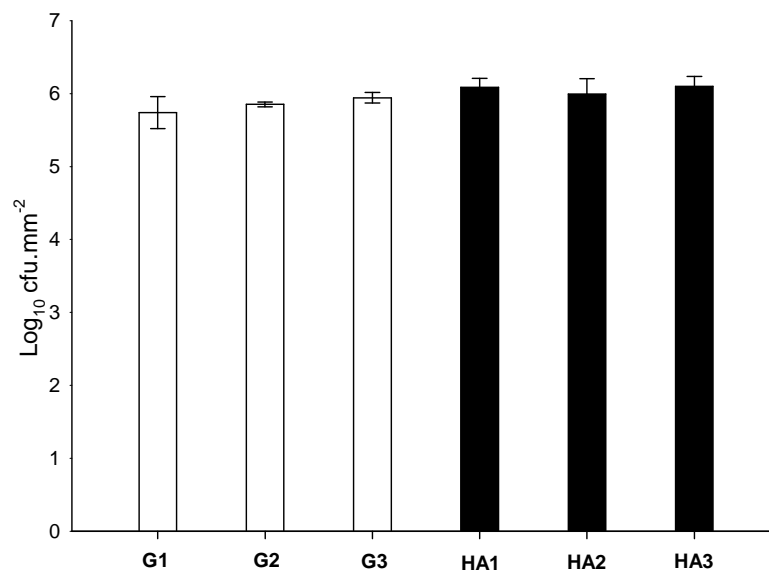
Figure 4.1: The effect of *N. subflava* cultures attachment on different substrata on total counts; G1, G2 and G3 (white bars) represent viable counts after incubation on glass substrata. HA1, HA2 and HA3 (black bars) represent viable counts after incubation on hydroxyapatite substrata. Data represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3).

Fig. 4.2 shows the ability *S. oralis* cultures to attach anaerobically for 48 hrs on both glass and HA surfaces. The viable counts of *S. oralis* resuspended biofilms grown previously on glass slides were not significantly different ($p > 0.05$). When HA was used as a surfaces for the growth of the same species, an insignificant difference ($p > 0.05$) of the viable counts was also detected. However, a significant difference ($p < 0.05$) of *ca.* 1.5 $\log_{10}$ cfu.mm^{-2} was shown with the counts of *S. oralis* grown on glass substrata compared to the counts of the microorganisms grown on HA discs.
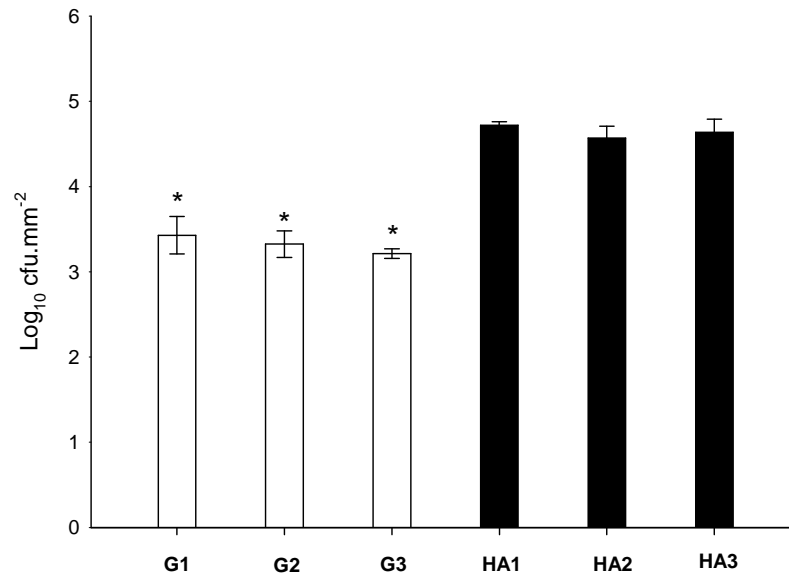
Figure 4.2: The effect of *S. oralis* cultures attachment on different substrata on total counts; G1, G2 and G3 (white bars) represent viable counts after incubation on glass substrata. HA1, HA2 and HA3 (black bars) represent viable counts after incubation on hydroxyapatite substrata. Data represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3). * above the open bar represents a significant change with respect to HA counts ($p < 0.05$).

Data presented in Fig. 4.3 shows a comparison of the effect of *S. sanguis* cultures attachment on both glass and HA surfaces on viable counts when grown anaerobically for 48 hrs. *S. sanguis* viable counts were slightly higher when biofilms were grown on glass compared to biofilms grown on HA substrata (Fig. 4.3). However, the slight increment in the viable counts was not considered to be statistically different ($p > 0.05$) between the counts after growth on the tested substrata.

In summary, there were no statistical differences between the viable counts of the suspended biofilms of each tested species when grown on the same substratum. Moreover, with the exception of *S. oralis*, the viable counts of the bacteria grown on glass were significantly different from the viable counts of the same bacteria grown on HA surfaces.
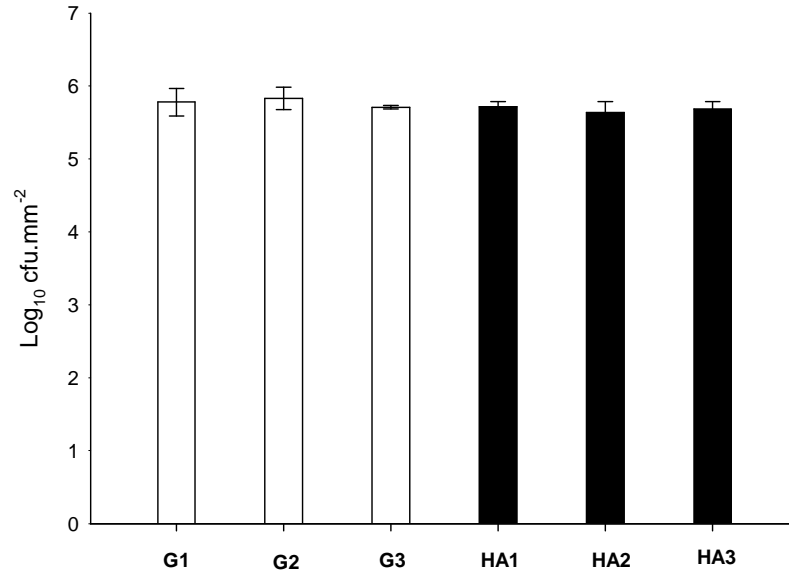
Figure 4.3: The effect of *S. sanguis* cultures attachment on different substrata on total counts; G1, G2 and G3 (white bars) represent viable counts after incubation on glass substrata. HA1, HA2 and HA3 (black bars) represent viable counts after incubation on hydroxyapatite substrata. Data represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3).

The ability to reproducibly grow biofilms from salivary microcosms on glass was compared to those grown on HA substrata. Fig. 4.4 illustrates the cells attachment following incubation of salivary samples on both glass and HA substrata, as measured by viable counts of the functional groups of the salivary microcosms. Fig. 4.4 (A), shows a significant difference in viable counts (p < 0.05) of 1.5 $\log_{10}$ cfu.mm^{-1} of total aerobes grown within salivary biofilms on glass substrata compared to viable counts of the same functional group grown on HA discs. Moreover, data presented in Fig. 4.4 (B-D), also show a significant difference in viable counts (p < 0.05) of *ca.* 1 $\log_{10}$ cfu.mm^{-1} of the rest of the bacterial functional groups grown within salivary biofilms on glass substrata compared to viable counts of the same functional group grown on HA discs.

In general, the data presented in Fig. 4.4 show the ability to produce reproducible biofilms on glass surfaces. However, in all trials viable counts of the functional

groups grown on glass substrata were significantly less than the viable counts of

the same functional groups grown HA substrata.
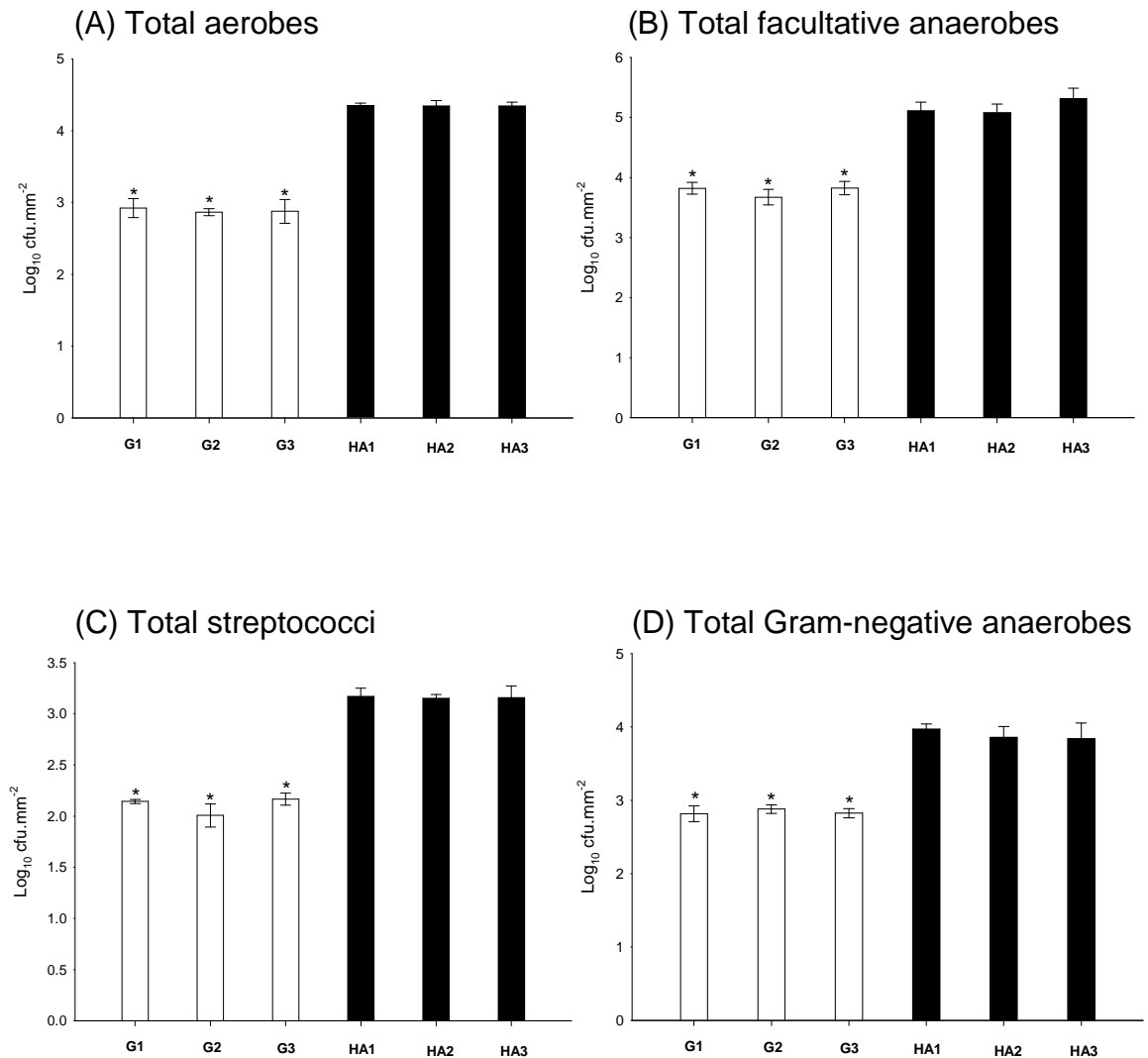
Figure 4.4: The effect of microorganisms attachment of whole saliva on different substrata on total counts; G1, G2 and G3 (white bars) represent viable counts after incubation on glass substrata. HA1, HA2 and HA3 (black bars) represent viable counts after incubation on hydroxyapatite substrata. Data represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3). * above the open bar represents a significant change with respect to HA counts (p < 0.05).

4.5.3 Antibacterial efficacy of various mouthwashes and their active component(s)

The hydroxyapatite disc model was used to evaluate and compare the antimicrobial efficacy of different oral antimicrobial agents against different functional bacterial groups in salivary microcosms. In this study commercially available mouthwashes were tested alongside their active ingredient(s). The mouthwashes and their respective antimicrobial agent(s) that were tested included; *i)* Listerine$^®$, containing the essential oils menthol, thymol, and eucalyptol, with methyl salicylate and alcohol also included in the formulation. *ii)* Neutrafluor$^®$, which contains the active ingredient NaF. *iii)* Plax$^®$overnight$^®$, containing the quaternary ammonium compound CPC. *iv)* Periogard$^®$, which contains the bisbiguanide CHX and *v)* Peroxyl$^®$, which includes the active ingredient H_2O_2 as an oxidising agent. It is important to note that the essential oils used in this study were either insoluble or slightly soluble in water but highly soluble in alcohol, therefore, alcohol (ethanol) at commercial concentration was used as a vehicle to solubilise them. The concentrations of the active components tested were equal to the concentrations of the same actives presented in the commercial counterpart mouthwash (Table 4.1).

Table 4.1: Active ingredients and their concentrations found in different tested commercially available mouthwashes.

Mouthwashes	Active ingredients	Concentrations of active ingredients
	Menthol*	0.042%
	Thymol*	0.064%
Listerine®	Eucalyptol*	0.092%
	Methyl salicylate*	0.060%
	Ethanol	26.1%
Neutrafluor®	Sodium fluoride	0.05%
Plax®overnight®	Cetyl pyridinum chloride	0.05%
Peroxyl®	Hydrogen peroxide	1.5%
Periogard®	Chlorhexidine	0.12%

* active ingredient was dissolved in 26.6% ethanol (as used in Listerine® formulation concentration) before being tested.

Data presented in Fig. 4.5 illustrate the effects of mouthwashes and their active components on the viable counts of total facultative anaerobe bacteria derived from salivary microcosms after one minute exposure. The mouthwashes Neutrafluor®, Peroxyl® and the active CPC did not show any significant reduction in the viable counts of the total facultative anaerobes (Fig. 4.5), whereas all other mouthwashes and their active components showed a statistically significant reduction in viable counts, the most prolific being Listerine® (4.5 $\log_{10}$ cfu.mm^{-1} reduction) and Periogard® (3 $\log_{10}$ cfu.mm^{-1} reduction).
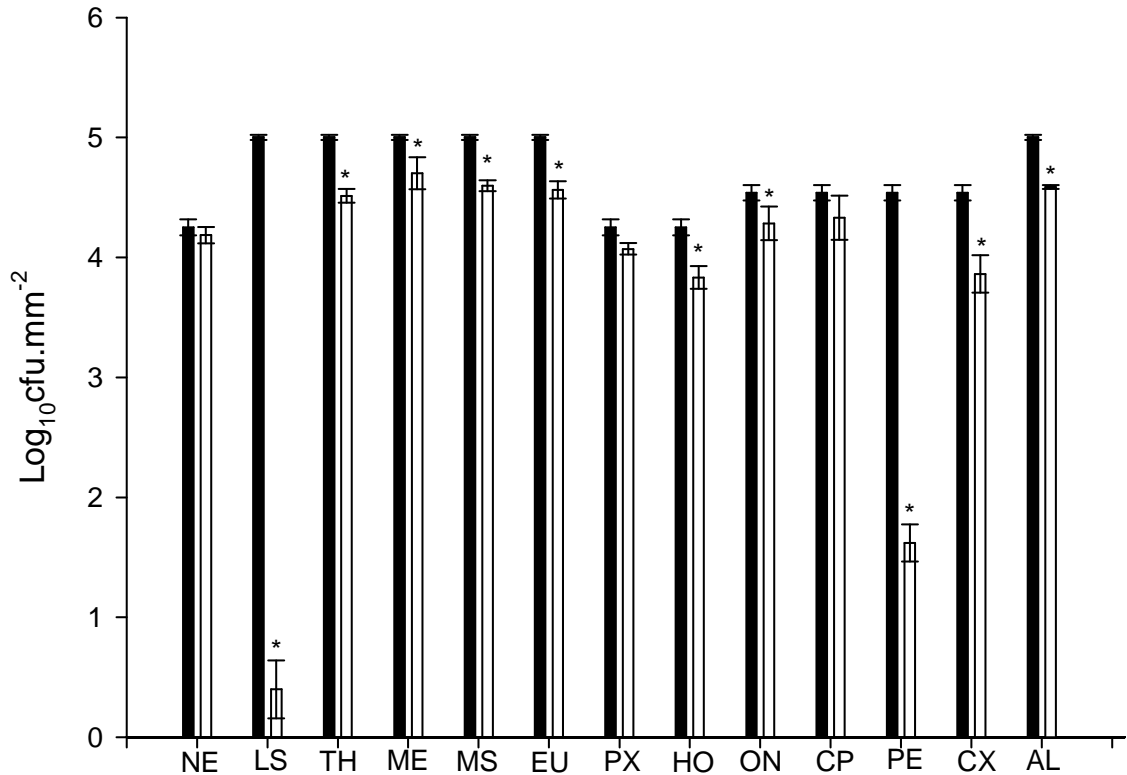
Figure 4.5: Effects of mouthwashes and actives on total facultative anaerobic viable bacterial counts of saliva-derived oral microcosms maintained on HA discs after one min. exposure; (NE) Neutrafluor[®], (LS) Listerine[®], (TH) thymol, (ME) menthol, (MS) methyl salicylate, (EU) eucalyptol, (PX) Peroxyl[®], (HO) H_2O_2, (ON) Plax[®]overnight[®], (CP) CPC, (PE) Periogard[®], (CX) CHX, (AL) alcohol. The solid black bars represent control counts (untreated microcosms) while the open bars represent the treated biofilm. Data represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3). * above the open bar represents a significant change with respect to control counts (p < 0.05).

The antimicrobial activities of the tested mouthwashes and actives against total anaerobes derived from salivary microcosms maintained on HA discs after one min exposure are illustrated in Fig. 4.6. A *ca.* 4 $\log_{10}$ cfu.mm^{-1} reduction of bacterial viable counts was detected after the one min. exposure of the salivary biofilm to Listerine[®] mouthwash. This reduction in viable counts was highly significant when compared to the control viable counts and other viable counts related to other mouthwashes and actives. Despite their statistically significant reduction of anaerobic viable counts, none of Listerine's[®] active ingredients showed a similar

reduction to the whole formulation. The most active component was thymol, showing a *ca.* 1 $\log_{10}$ cfu.mm^{-1} reduction in bacterial viable counts.
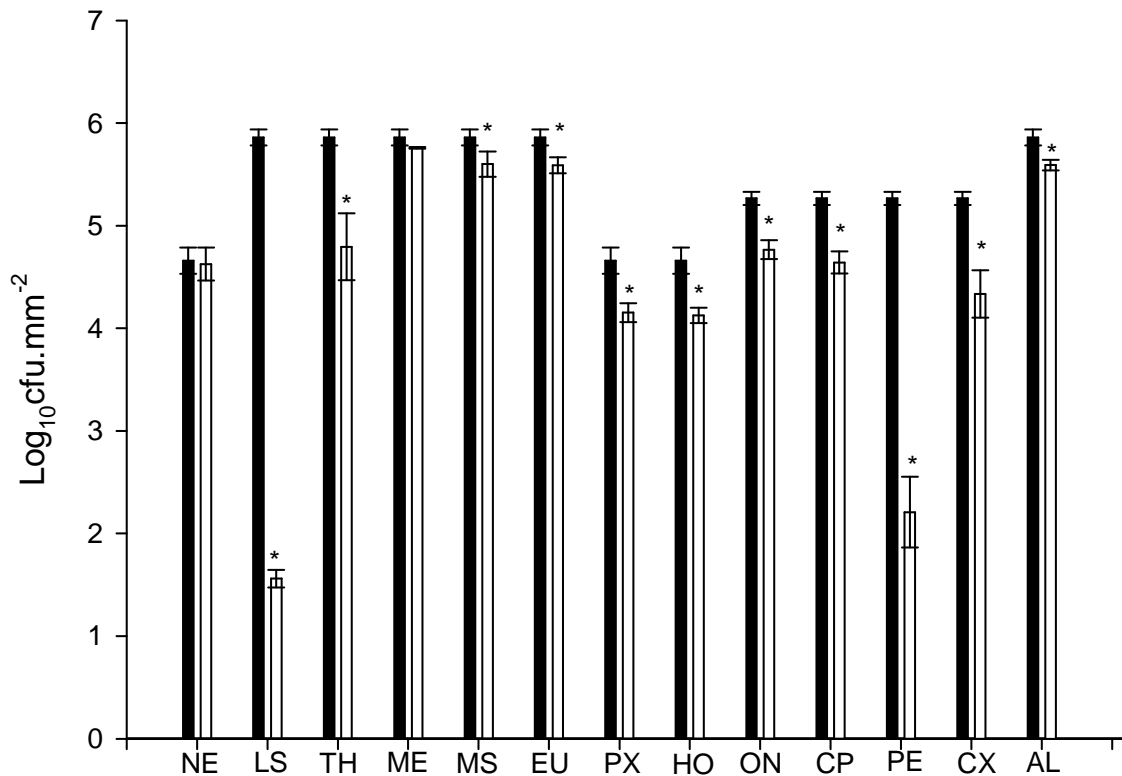
Figure 4.6: Effects of mouthwashes and actives on total anaerobic viable bacterial counts of saliva-derived oral microcosms maintained on HA discs after one min. exposure; (NE) Neutrafluor®, (LS) Listerine®, (TH) thymol, (ME) menthol, (MS) methyl salicylate, (EU) eucalyptol, (PX) Peroxyl®, (HO) H_2O_2, (ON) Plax®overnight®, (CP) CPC, (PE) Periogard®, (CX) CHX, (AL) alcohol. The solid black bars represent control counts (untreated microcosms) while the open bars represent the treated biofilm. Data represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3). * above the open bar represents a significant change with respect to control counts ($p < 0.05$).

Other actives in the study such as CHX showed a significant reduction in cell counts (*ca.*1 $\log_{10}$ cfu.mm^{-1}) compared to the control. However, this antibacterial activity was considered less than the activity of its commercial mouthwash counterpart Periogard®, which showed a reduction of *ca.* 3 $\log_{10}$ cfu.mm^{-1} compared to the controls. The rest of the tested actives and their counterpart mouthwashes showed a reduction of approximatly 0.5 $\log_{10}$ cfu.mm^{-1} of the

bacterial viable counts compared to their related controls, except for Neutrafluor[®] which, did not show any significant reduction on cell numbers.

The antimicrobial efficacies against other bacterial functional groups in the salivary-derived biofilm were also tested. Fig. 4.7 illustrates the antimicrobial activity of different actives and their related mouthwashes on the viable counts of total Gram-negative anaerobic bacterial groups derived from salivary microcosms attained on HA discs after one min exposure. As previously shown with other functional bacterial groups, Listerine[®] mouthwash demonstrated a high antimicrobial efficacy which was represented by *ca.* 5 $\log_{10}$ cfu.mm^{-1} reduction in the viable counts of the related Gram-negative anaerobes compared to the control (Fig. 4.7). Thymol, as one of Listerine's[®] actives, also showed a significant reduction in viable counts of the same bacterial functional groups (3 $\log_{10}$ cfu.mm^{-1}; Fig. 4.7). Data presented in Fig. 4.7 also indicate that the rest of the active ingredients of Listerine[®] did not show the same potent antibacterial efficacy of Listerine[®] itself or thymol's when compared to the control. Fig. 4.7 also demonstrates the antibacterial efficacy of other formulations such as Peroxyl[®] and its active agent H_2O_2, both of which showed a reduction of the viable counts of the related Gram-negative anaerobes. This reduction was calculated as *ca.* 2.5 and 2.25 $\log_{10}$ cfu.mm^{-1} respectively. The mouthwash Periogard[®] and its active CHX showed reduction values of *ca.* 2.5 and 2 $\log_{10}$ cfu.mm^{-1} respectively (Fig. 4.7). The lowest antibacterial activity against the Gram-negative anaerobes of the salivary microcosm was represented by the high viable counts of these species after exposure to the mouthwashes Neutrafluor[®], Plax[®]overnight[®] and its active CPC (Fig. 4.7).
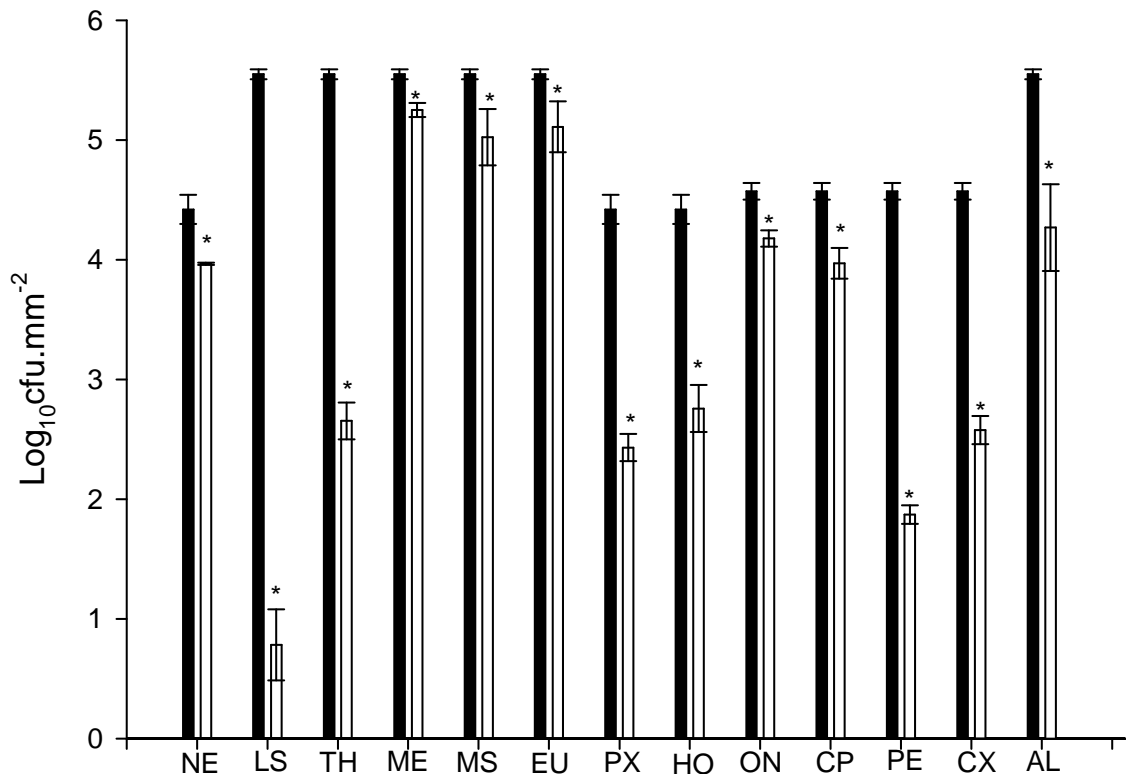
Figure 4.7: Effects of mouthwashes and actives on total Gram-negative anaerobic viable bacterial counts of saliva-derived oral microcosms maintained on HA discs after one min. exposure; (NE) Neutrafluor®, (LS) Listerine®, (TH) thymol, (ME) menthol, (MS) methyl salicylate, (EU) eucalyptol, (PX) Peroxyl®, (HO) H_2O_2, (ON) Plax®overnight®, (CP) CPC, (PE) Periogard®, (CX) CHX, (AL) alcohol. The solid black bars represent control counts (untreated microcosms) while the open bars represent the treated biofilm. Data represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3). * above the open bar represents a significant change with respect to control counts (p < 0.05).

The effects of one min. exposure to different actives and mouthwashes on total streptococci is presented in Fig. 4.8. A significant reduction of the streptococci functional group was observed after treatment with Listerine®. It can be seen from Fig. 4.8 that Listerine's® active ingredients individually failed to show the same reduction in counts as that of the commercial product itself. Statistical analysis confirmed that there was no significant reduction in the counts after treatment with Neutrafluor®, Peroxyl®, Plax®overnight® and the active ingredients represented by them (Fig. 4.8). Treatment with Periogard® and its active CHX showed a reduction in viable counts of *ca.* 2.5 and 1 log_{10} cfu.mm^{-1} respectively (Fig. 4.8).
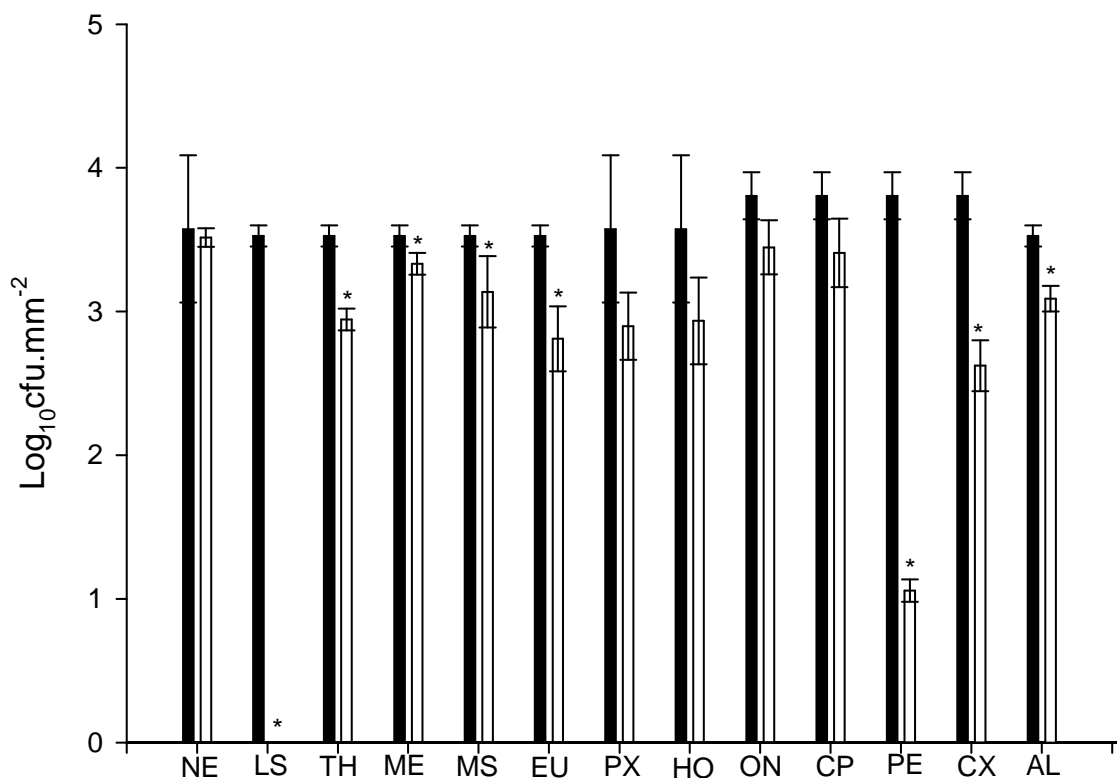
Figure 4.8: Effects of mouthwashes and actives on total streptococci viable bacterial counts of saliva-derived oral microcosms maintained on HA discs after one min. exposure; (NE) Neutrafluor[®], (LS) Listerine[®], (TH) thymol, (ME) menthol, (MS) methyl salicylate, (EU) eucalyptol, (PX) Peroxyl[®], (HO) H_2O_2, (ON) Plax[®]overnight[®], (CP) CPC, (PE) Periogard[®], (CX) CHX, (AL) alcohol. The solid black bars represent control counts (untreated microcosms) while the open bars represent the treated biofilm. Data represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3). * above the open bar represents a significant change with respect to control counts ($p < 0.05$).

In summary, treatment of saliva-derived microcosms with Listerine[®] has successfully shown a high reduction in bacterial counts which was manifested against total facultative anaerobes, total aerobes, Gram-negative and total streptococci (Figs. 4.5-4.8). This reduction was observed to be greater than the viable count reductions of the other mouthwashes and their actives that were tested. Moreover, the reduction of viable counts by Listerine[®] was significantly higher than that of Listerine[®] actives used individually. Therefore, further investigations into detecting the mechanisms of action of Listerine[®] formulation itself and its actives individually and in combination were conducted. The ability of

the bacterial biomass to detach from its substrata after exposure to Listerine$^{®}$ and/or its actives individually was also examined as described (Section 2.14). The antibacterial effect of Listerine$^{®}$ formulation was investigated microscopically as described (Section 2.15).

4.5.4 Investigations into the effects of Listerine$^{®}$ and its active ingredients on biomass detachment

Crystal violet assays were used to investigate the ability of Listerine$^{®}$ and its active agenst to perturb the attachment integrity of artificial plaque biomass. As previously described in Section 2.14, salivary-derived bacterial biomass were grown on peg devices both aerobically and anaerobically for 48 hrs before exposure to the mouthwash and its active agents individually for one min. duration. Crystal violet staining of the biofilm material remaining on the peg devices was compared spectrophotometrically after the crystal violet was re-solubilised. A high optical density would imply to a high artificial plaque mass remaining on the peg device and *vice versa*. Data displayed in Fig. 4.9 and Fig. 4.10 illustrate the effects caused by Listerine$^{®}$ and its active ingredients on the optical densities of crystal violet stained-artificial plaque after one min exposure. According to statistical analysis the crystal violet optical densities of all stained microcosms did not show any significant difference compared to control optical density values (Fig. 4.9 and Fig. 4.10). The only significant difference was observed in the optical densities of the negative control values, where this difference was considered to be due to the absence of growing bacteria in the sterilised saliva representing negative controls (Fig. 4.9 and Fig. 4.10).
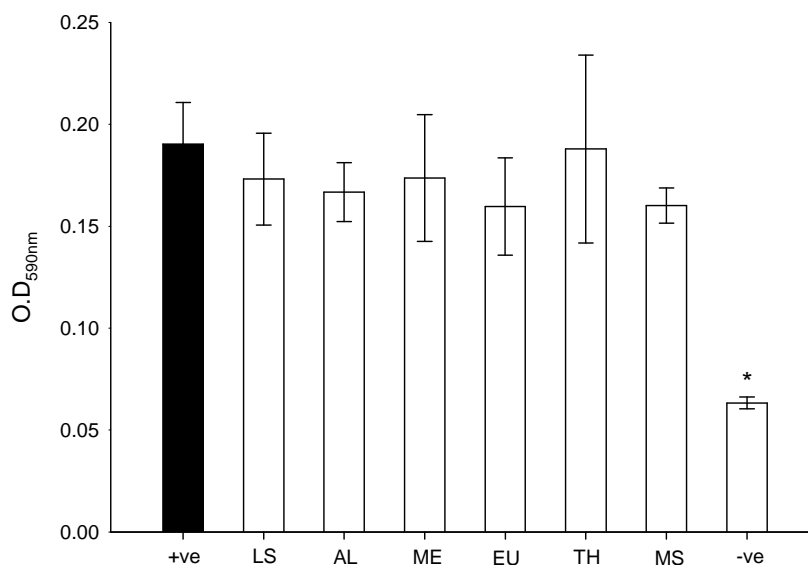
Figure 4.9: Effects of Listerine® mouthwash and its active ingredients on crystal violet O.D of stained salivary-derived microcosms maintained aerobically on peg devices after 1 min. exposure; (+ve) positive control, (LS) Listerine®, (AL) Alcohol, (ME) menthol, (EU) eucalyptol, (TH) thymol, (MS) methyl salicylate, (-ve) negative control. The solid black bar represents the control O.D (untreated microcosms) while the open bars represent the treated biofilm. Data represent mean values of eight replicates; error bars represent standard errors of the mean (n=8). * above the open bar represents a significant change with respect to control O.D ($p < 0.05$).

It was also observed from both Figs. (4.9 and 4.10), that all the optical density values of crystal violet representing the remaining biomass attached on pegs was higher in aerobic conditions. These differences in optical densities were also consistent with positive control values representing the untreated microcosms. This consistency is more likely to represent the magnitude of biomass growth in aerobic conditions rather than being a resemblance of ability of the E.O's to detach the plaque. The study design incubation period of the anaerobic plaque (48 hours), the slow growth of the different taxonomic species (strict and facultative anaerobes) and the plastic substratum of the peg material might be causative factors for a lower bacterial biomass on pegs incubated anaerobically which represent low optical densities of crystal violet values.
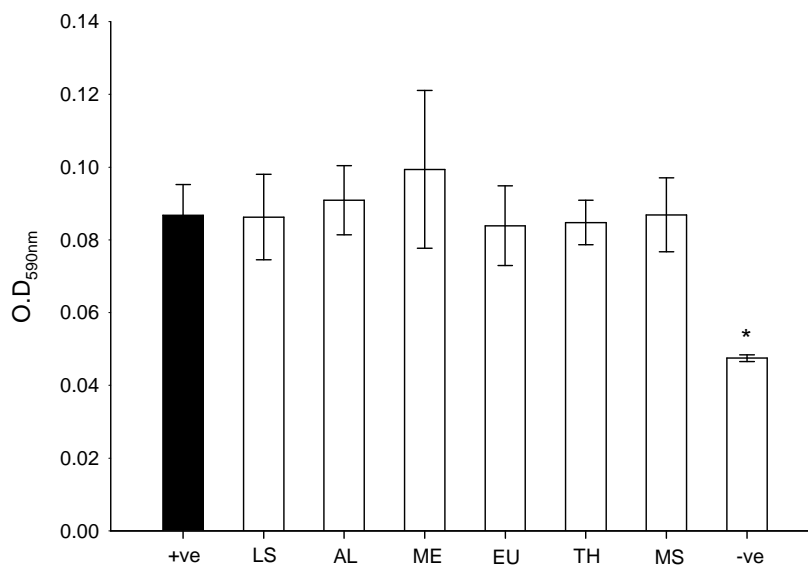
Figure 4.10: Effects of Listerine® mouthwash and its active ingredients on crystal violet O.D of stained salivary-derived microcosms maintained anaerobically on peg devices after 1 min. exposure; (+ve) positive control, (LS) Listerine®, (AL) Alcohol, (ME) menthol, (EU) eucalyptol, (TH) thymol, (MS) methyl salicylate, (-ve) negative control. The solid black bar represents the control O.D (untreated microcosms) while the open bars represent the treated biofilm. Data represent mean values of eight replicates; error bars represent standard errors of the mean (n=8). * above the open bar represents a significant change with respect to control O.D ($p < 0.05$).

4.5.5 A comparative investigation of the combinatorial antibacterial efficacies of Listerine® mouthwash active components

An *in vitro* approach was used to investigate the antibacterial activity of Listerine® active ingredients. The HDM was used to investigate the active agents combinatorial antibacterial effect in the following possible combinations; *i)* eucalyptol-menthol combination (EU-ME), *ii)* thymol-menthol combination (TH-ME), *iii)* methyl salicylate-menthol combination (MS-ME), *vi)* thymol-eucalyptol combination (TH-EU), *v)* methyl salicylate-eucalyptol combination (MS-EU), and *iv)* thymol-methyl salicylate combination (TH-MS). In all cases the combinatorial actives concentrations were equal to the concentrations found in the product and were dissolved in 26.1% ethanol to mimic the product formulation and to enhance the solubility of the ingredients. The antibacterial efficacy was evaluated by comparing viable bacterial counts of the saliva-derived microcosm that had been

163

maintained on HA discs and treated one min. with the combinatorial actives (Section 2.12).

Fig. 4.11 shows the effects a 1min. exposure to Listerine® mouthwash and its combinatorial actives on viable bacterial counts of total facultative anaerobes of saliva-derived oral microcosms maintained on HA discs.
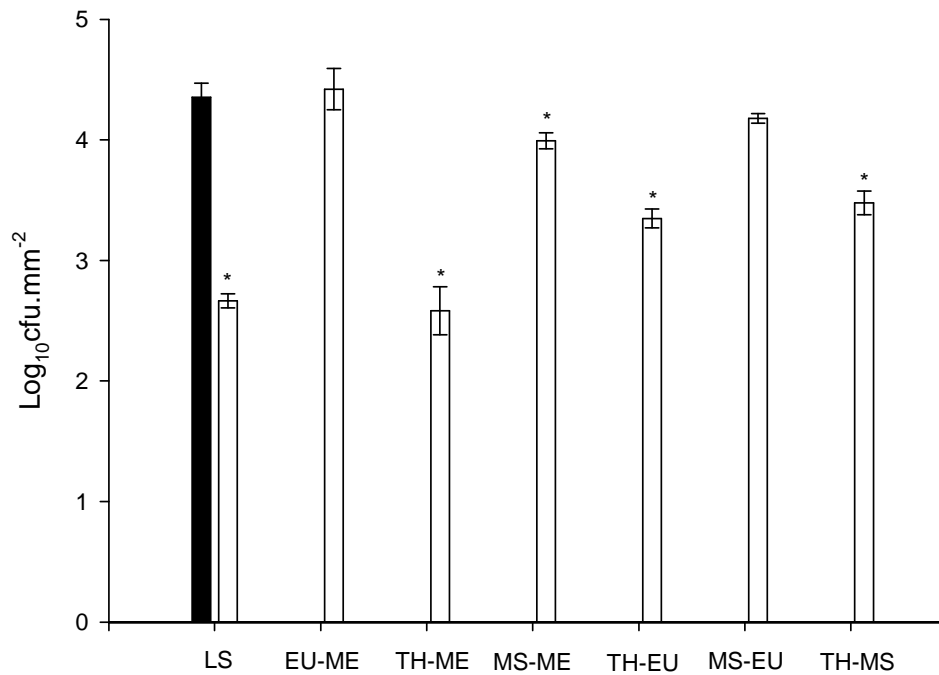
Figure 4.11: Effects of Listerine® mouthwash and its combinatorial actives on total facultative anaerobic viable bacterial counts of saliva-derived oral microcosms maintained on HA discs after one min. exposure; (LS) Listerine®, (EU-ME) eucalyptol-menthol combination, (TH-ME) thymol-menthol combination, (MS-ME), methyl salicylate-menthol combination, (TH-EU) thymol-eucalyptol combination, (MS-EU) methyl salicylate-eucalyptol combination, (TH-MS) thymol-methyl salicylate combination. The solid black bar represents control counts (untreated microcosms) while the open bars represent the treated biofilm. Data represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3). * above the open bar represents a significant change with respect to control counts (p < 0.05).

It was observed from the data presented in Fig. 4.11 that all combinatorial actives except that of EU-ME promoted statistically significant reductions in viable counts compared to the control. Both Listerine® and the TH-ME combination were substantially more effective in reducing cell number than the rest of the tested combinations (Fig. 4.11). Statistical analysis showed that the reduction in viable counts caused by Listerine® and the TH-ME actives combination was not

significantly different; hence, both the formulation itself and TH-ME combination show a similar antimicrobial effectiveness (*ca.*1.5 $\log_{10}$ cfu.mm^{-1} reductions).

Fig. 4.12 illustrates the effectiveness of Listerine® mouthwash and its combinatorial actives on the reduction in numbers of total anaerobic bacteria. The data presented in the same figure shows that TH-EU and TH-MS actives combination were equally effective in promoting the reduction of bacterial CFU's (*ca.*1.5 $\log_{10}$ cfu.mm^{-1}). TH-ME had a modestly better antibacterial efficacy compared to the Listerine® formulation (Fig. 4.12). Whilst EU-ME failed to show any significant reduction in viable counts compared to the control, MS-EU showed a minor reduction followed by the CFU reduction promoted by MS-ME combination (Fig. 4.12).
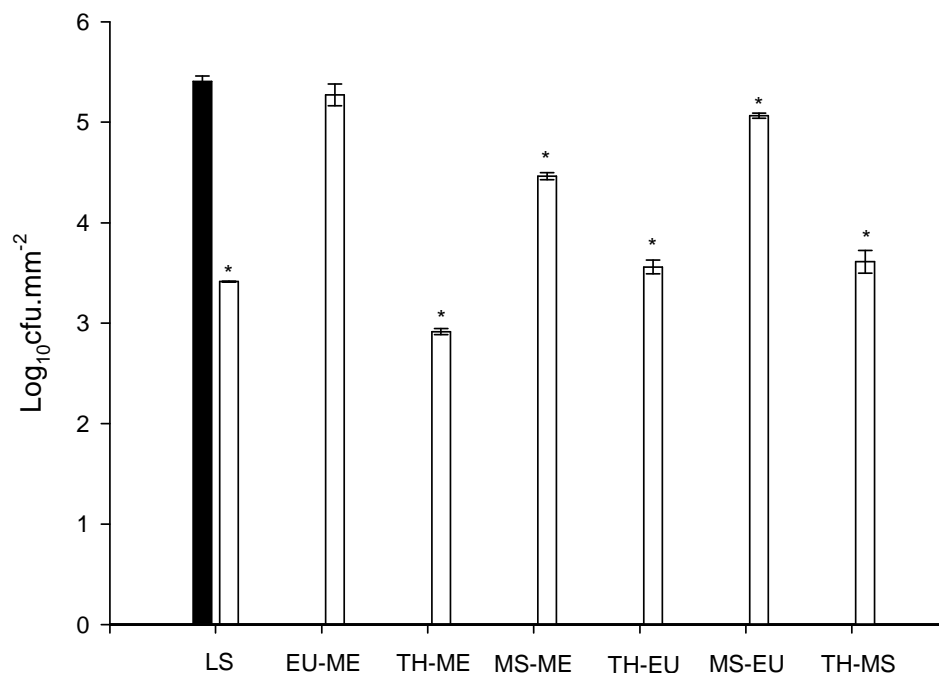
Figure 4.12: Effects of Listerine® mouthwash and its combinatorial actives on total anaerobic viable bacterial counts of saliva-derived oral microcosms maintained on HA discs after one min. exposure; (LS) Listerine®, (EU-ME) eucalyptol-menthol combination, (TH-ME) thymol-menthol combination, (MS-ME), methyl salicylate-menthol combination, (TH-EU) thymol-eucalyptol combination, (MS-EU) methyl salicylate-eucalyptol combination, (TH-MS) thymol-methyl salicylate combination. The solid black bar represents control counts (untreated microcosms) while the open bars represent the treated biofilm. Data represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3). * above the open bar represents a significant change with respect to control counts (p < 0.05).

The results shown in Fig. 4.13 provide a clear representation of the antibacterial effectiveness of Listerine® mouthwash and its combinatorial actives on total Gram-negative anaerobic viable bacterial counts. The viable counts reduction efficacies of the combinations TH-ME, TH-EU and TH-MS were slightly better than Listerine® as a whole. Listerine® mouthwash itself showed a substantial antibacterial effectiveness by achieving a reduction of *ca.* 2.5 $\log_{10}$ cfu.mm^{-1} (Fig. 4.13). Viable counts reduction affected by exposure of biofilms to EU-ME combination were slightly more than the control, whereas MS-EU combination did not show any statistical difference from the control. The combination of MS-ME was effective in causing a *ca.* 1 $\log_{10}$ cfu.mm^{-1} reduction of CFU's as shown in Fig. 4.13.
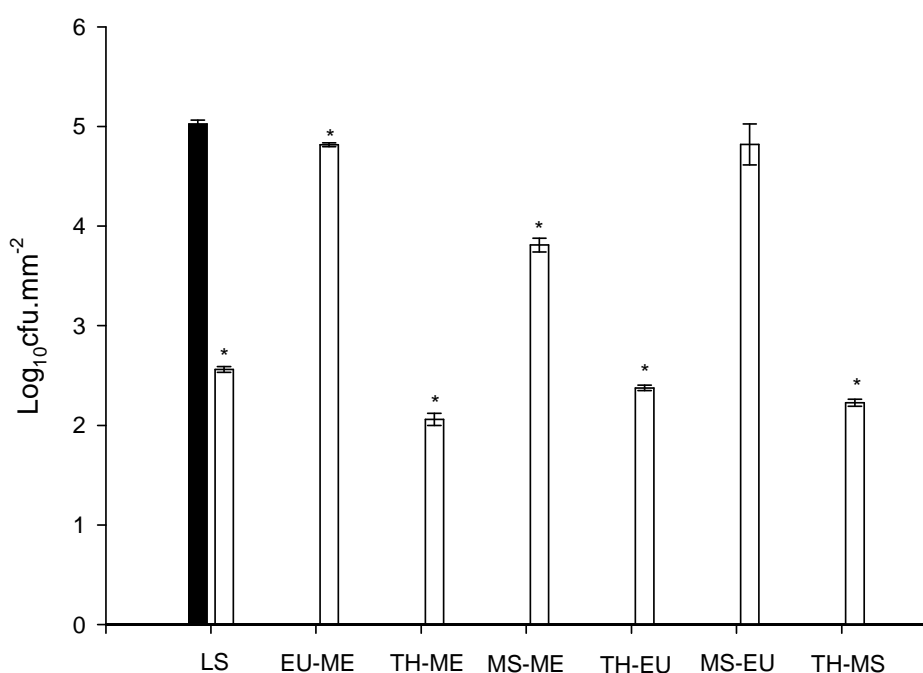
Figure 4.13: Effects of Listerine® mouthwash and its combinatorial actives on total Gram negative anaerobic viable bacterial counts of saliva-derived oral microcosms maintained on HA discs after one min. exposure; (LS) Listerine®, (EU-ME) eucalyptol-menthol combination, (TH-ME) thymol-menthol combination, (MS-ME), methyl salicylate-menthol combination, (TH-EU) thymol-eucalyptol combination, (MS-EU) methyl salicylate-eucalyptol combination, (TH-MS) thymol-methyl salicylate combination. The solid black bar represents control counts (untreated microcosms) while the open bars represent the treated biofilm. Data represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3). * above the open bar represents a significant change with respect to control counts (p < 0.05).

The effectiveness of Listerine® mouthwash and paired combination of active compounds against total streptococci CFU's was demonstrated in Fig. 4.14. Active ingredient combinations of EU-ME, MS-ME and MS-EU did not show any significant statistical difference in promoting a viable counts reduction compared to the control counts (Fig. 4.14). Statistical analyses have shown that the antimicrobial efficacy of both Listerine® mouthwash and the combination of TH-ME were not significantly different to each other, but were significantly different to the control values (*ca.* 2 $\log_{10}$ cfu.mm^{-1} reduction). Statistical analyses have also shown that the antimicrobial efficacy of both TH-EU and TH-MS combinations was not significantly different when compared to each other. Moreover, TH-EU and TH-MS combinations had a modest effect on reducing the total streptococci counts (*ca.* 1 $\log_{10}$ cfu.mm^{-1} reduction) as demonstrated in Fig. 4.14.

In summary, the presence of the essential oil thymol in any of the tested formulations led to a better reduction in the differential viable bacterial counts compared to other active ingredients formulations (Figs. 4.11-4.14). The paired combination of TH-ME was as effective as, or slightly better than, Listerine® mouthwash in reducing the CFU's of different bacterial functional groups. Therefore, the combination of thymol and menthol were further tested for any potential synergy against pure oral bacterial species in the incoming section of the results.
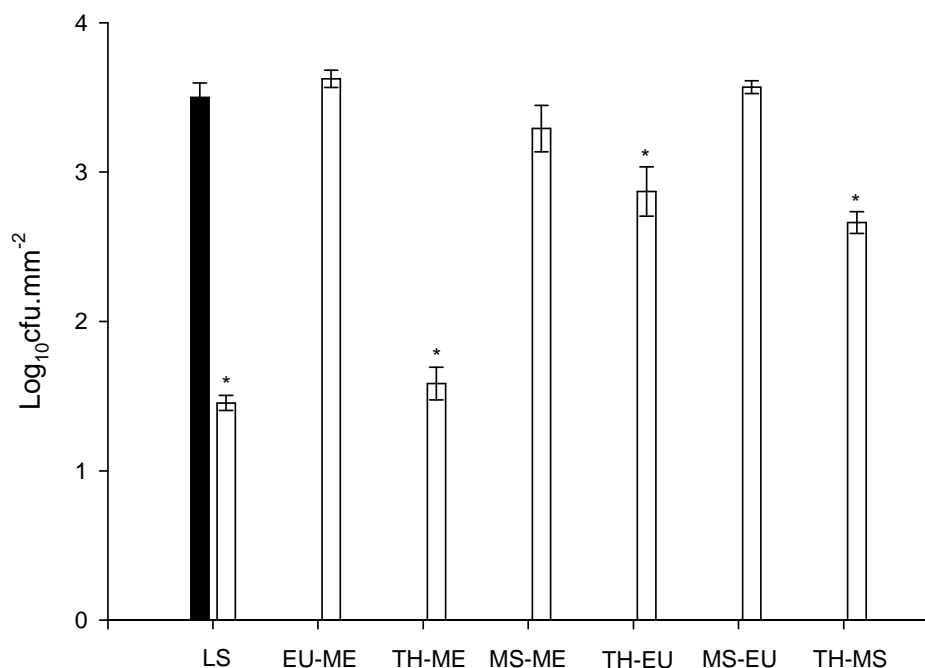
Figure 4.14: Effects of Listerine® mouthwash and its combinatorial actives on total streptococci viable bacterial counts of saliva-derived oral microcosms maintained on HA discs after one min. exposure; (LS) Listerine®, (EU-ME) eucalyptol-menthol combination, (TH-ME) thymol-menthol combination, (MS-ME), methyl salicylate-menthol combination, (TH-EU) thymol-eucalyptol combination, (MS-EU) methyl salicylate-eucalyptol combination, (TH-MS) thymol-methyl salicylate combination. The solid black bar represents control counts (untreated microcosms) while the open bars represent the treated biofilm. Data represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3). * above the open bar represents a significant change with respect to control counts ($p < 0.05$).

4.5.6 Into investigating a potential combinatorial antimicrobial activity between thymol and menthol essential oils

A checkerboard microdilution method was used to investigate the presence of a potential synergistic effect between the essential oils thymol and menthol. According to the data previously presented in section 4.5.5, the combination of both oils solubilised in 26.1% alcohol was as active as or slightly better than the complete product. In order to compare their synergistic effect, both actives were tested individually before being tested in combination against various oral bacterial species as previously described in section 2.10. The interactions between the two essential oils were classified as synergistic, additive, or antagonistic on the basis of the fractional inhibitory concentration (FIC) index. The FIC index is the sum of

the FICs of each tested agent and the FIC is defined as the MIC of each antimicrobial when used in combination divided by the MIC of the antimicrobial when used alone. As previously described in Section 3.4.3.1, the interaction was defined as synergistic if the FIC index was <1, additive if the FIC index was 1.0, sub-additive if the FIC index was between 1.0 and 2.0, indifferent if the FIC index was 2, and antagonistic if the FIC index >2 (Berenbaum 1978; Hall *et al.* 1983; Scott *et al.* 1999; Canton *et al.* 2005). The combination of both essential oils was demonstrated against various tested species as shown in Table 4.2.

In accordance to the FIC index values presented in Table 4.1, both essential oils (thymol and menthol) were shown to possess a synergistic antimicrobial activity against most of the tested oral species. However, the MIC values for thymol in the combinatorial solution ranged from ($10\mu g.ml^{-1}$-$0.16mg.ml^{-1}$), whereas in Listerine[®] the MIC values of thymol active ingredient ranged from ($5\mu g.ml^{-1}$-$0.32mg.ml^{-1}$). Menthol MIC values have also shown to be lower in the combination ($30\ \mu g.ml^{-1}$-$70\mu g.ml^{-1}$) compared to the mouthwash itself ($30\mu g.ml^{-1}$-$0.21mg.ml^{-1}$). The higher MIC values of Listerine[®] compared to its actives combination indicate its lower antibacterial activity. This lower activity could be caused by the presence of various active and inactive ingredients in the mouthwash solution which might antagonise the antibacterial activity of thymol and menthol synergy. Several trials to investigate the nature of antagonising ingredients by the checkerboard method failed (data not presented) due to the inability to produce a clear solution when a fourth active ingredient was introduced.

4.5.7 Microscopic evaluation of Listerine® mouthwash on artificial plaque

The capability of Listerine® mouthwash to penetrate and/or detach a salivary-derived plaque biofilm in addition to its antibacterial action was tested. Glass slides were used as substrata for the biofilm. Incubation period and mouthwash exposure time were previously described in (Section 2.15).
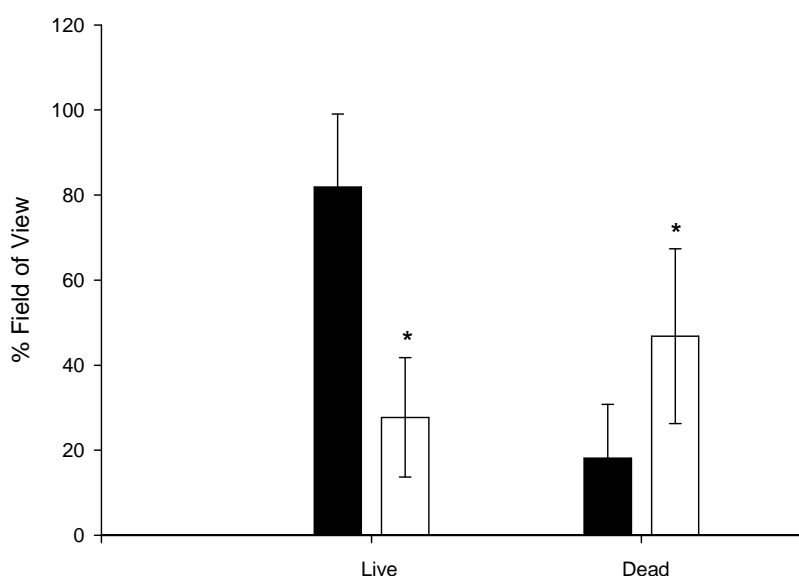
Figure 4.15: Effects of Listerine® mouthwash on attachment and viability of saliva-derived oral microcosm as determined by LIVE/DEAD *Bac*Light assay. Data are means ± SD from 2 glass slides, each of which counted ten random fields of view. Filled bars, control (untreated microcosm); open bars, Listerine® treated microcosm. * above the open bar represents a significant change with respect to control counts (p < 0.05).

Fig. 4.15 shows data for numbers of individual live and dead cells developed in control and Listerine®-exposed environments with salivary bacterial consortia maintained on glass slides. Brief exposure (1 min) to the mouthwash significantly reduced the number of live cells compared to untreated controls (Fig. 4.15). The increase in the number of dead microorganism was statistically significant after exposure to Listerine® compared to untreated biofilms (Fig. 4.15). Measurements of the live/dead cells, however, do not necessarily correlate only with lethality of the mouthwash, as the data represented in Fig. 4.15 show that the sum of the

treated live and dead cells was still lower than 100% field of view. This observation was in apparent agreement with the images presented in Fig. 4.16. The number and the aggregations of the scored live and dead microorganisms after Listerine® exposure were lower per field of view compared to control. A detachment of the cells and a washing effect is proposed.
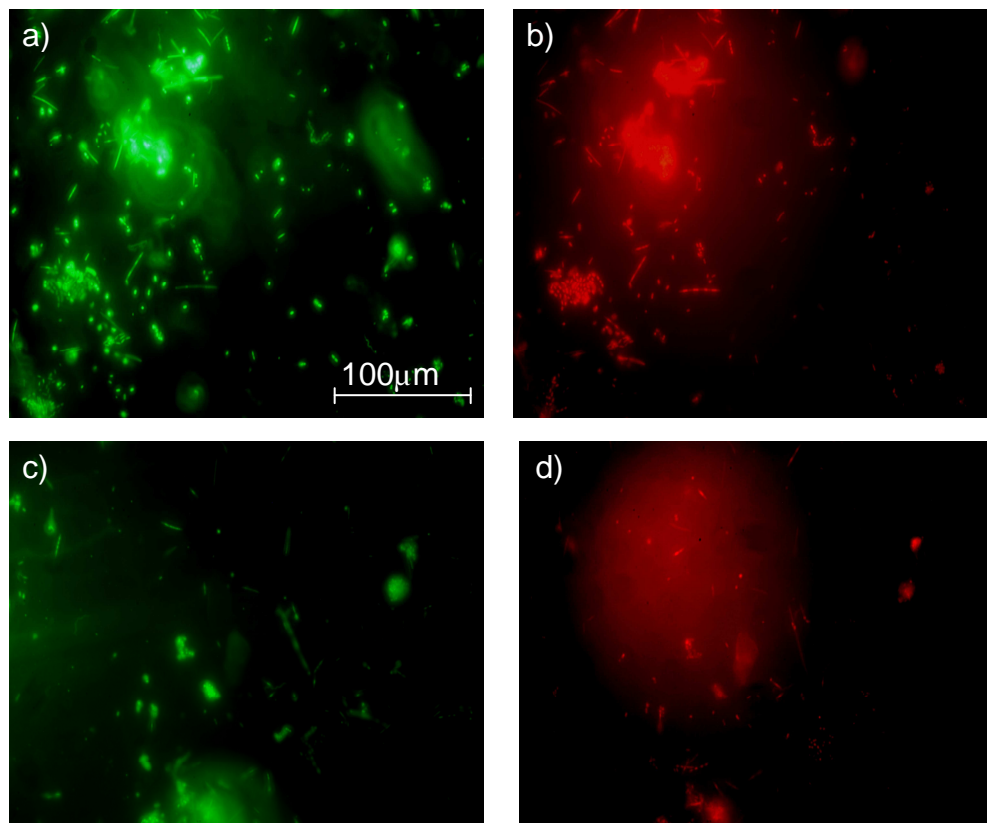
Figure 4.16: Epifluorescence microscope images showing LIVE/DEAD-stained saliva-derived plaque formed on glass slides. (a) Control (live); (b) control (dead); (c) treated (live); (d) treated (dead). Total magnification: x1000.

Table 4.2: *In vitro* interactions between thymol and menthol on various axenic species of the oral microcosm indicated by the fractional inhibitory concentration index (FIC) method.

Organism	Thymol (mg.ml⁻¹)[a]		Menthol (mg.ml⁻¹)[b]		Thymol (mg.ml⁻¹)[c]		Menthol (mg.ml⁻¹)[d]		Checkerboard FIC index	Interpretation	Listerine® %		Thymol (mg.ml⁻¹)[e]		Menthol (mg.ml⁻¹)[f]	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC			MIC	MBC	MIC	MBC	MIC	MBC
S. oralis	0.16	0.64	0.21	> 0.42[g]	0.005	0.005 0.005 (0.01)[h]	0.003	0.003	0.046	S[i]	12.5	50	0.08	0.32	0.05	0.21
S. mutans	0.32	0.32	> 0.42	> 0.42	0.04 0.04 (0.01)	(0.01) (0.08) (0.04)	0.003	0.003	0.09	S	25 (50)	50	0.16	0.32	0.1	0.21
P. oralis	0.32	0.32	0.105	0.105	0.08	0.08	0.007	0.007	0.3	S	50	50	0.32	0.32	0.21	0.21
S. sanguis	0.16	0.16	0.42	0.42	0.005	0.005	0.003	0.003	0.039	S	12.5	25	0.08	0.16	0.05	0.1
L. rhamnosus	0.32	0.32	> 0.42	> 0.42	0.16	0.32	0.003	0.003	0.5	MS[j]	50	50	0.32	0.32	0.21	0.21
P. gingivalis	0.16	0.16	0.007	0.007	0.001	0.001	0.007	0.007	1.008	SA[k]	50	50	0.32	0.32	0.21	0.21
N. subflava	0.02	0.08	0.05 0.05 (0.1)	0.1 0.1 (0.2)	0.005	0.005	0.003	0.003	0.3	S	0.78	3.1 (1.56)	0.005	0.02	0.003	0.013

[a,b]; MICs and MBCs for essential oils tested individualy, [c,d]; MICs and MBCs for essential oils tested in combination, [e]; concentration of thymol represented in Listerine® [f]; concentration of menthol represented in Listerine®, [g]; value greater than the tested concentration, [h]; different value, [i]; synergy, [j]; marginal synergy, [k]; sub-additive.

4.6 Discussion

This chapter compared the antibacterial efficacy of a number of commercially available mouthwashes and their active ingredients, namely Listerine[®], Periogard[®], Plax[®]overnight[®], Peroxyl[®] and Neutrafluor[®]. The comparative experiments were performed using a hydroxyapatite disc as an *in vitro* biofilm model, first described by Guggenheim in 1999. The results suggest that Listerine[®] mouthwash showed a higher reduction in the total counts of various bacterial functional groups after one min. exposure compared to other tested mouthwashes (Neutrafluor[®], Plax[®]overnight[®], Periogard[®] and Peroxyl[®]); (Figs. 4.5, 4.6, 4.7 and 4.8).

Interestingly, the individual active ingredients of Listerine[®] mouthwash, namely menthol, thymol, eucalyptol, methyl salicylate and ethanol at formulation concentrations showed only a modest reduction in the viable counts of bacterial functional groups, suggesting combinatorial action of two or more of the components (Figs. 4.5, 4.6, 4.7 and 4.8). Although Periogard[®] mouthwash showed a reduction in total counts, its efficacy was not as great as that of Listerine[®]. These findings are in agreement with Pan *et al* who suggested that the rapid killing activity of Listerine[®] mouthwash was attributed to its ability to penetrate plaque and damage bacterial cellular membrane (Pan *et al.* 2000). Due to this antibacterial efficacy, Listerine[®] mouthwash was further investigated. Data presented in the HDM study could not give a full explanation of the mechanism by which either Listerine[®] or its active ingredients exert their antimicrobial efficacy. The HDM study data provided information on bacterial functional groups counts reduction caused by Listerine[®] exposure. Again, this reduction could be a result of the biofilm penetrative activity of Listerine[®], leading to biofilm removal from the

substrata and not necessarily kill. Therefore, other approaches were conducted in order to provide more information regarding the antibacterial mode of action Listerine® and its active ingredients. Biofilm detachment investigations (crystal violet assays) were conducted to see whether Listerine® and/or any of its active ingredients were responsible for plaque removal. Results did not show any significant difference between the optical densities for treated and non-treated biofilms, for Listerine® or any of its active ingredients that were tested (Figs. 4.9 and 4.10). These results suggest that neither Listerine® nor any of its active ingredients had any impact on biofilms detachment from the treated plaque-containing pegs. Despite showing a negative removal impact of biofilms in crystal violet assays, microscopic evaluation experiments of LIVE/DEAD *Bac*Light assays were conducted to investigate both the lethality and the removal activity presence of Listerine® formulation (Figs. 4.15 and 4.16). It is suggested from the results of LIVE/DEAD *Bac*Light assays that Listerine® mouthwash was able to rapidly kill microorganisms of the salivary bacterial consortia that were maintained on the glass slides. The data showed that *ca.* 50% of the Listerine®-treated oral biofilm was killed, *ca.* 30% live and *ca.* 20% absent or detached from its substrata. Data from the LIVE/DEAD *Bac*Light assays suggest that a removal activity of biofilms after brief exposure to the mouthwash could occur. The effect of surface chemistry and topography on microbial attachment has been previously studied (Cunliffe *et al.* 1999). However, the hydrophilic property of glass surfaces make them more "hygienic" (Boyd *et al.* 2000; Verran *et al.* 2008). Therefore, the differences in the topographic properties of the tested glass slides in the LIVE/DEAD *Bac*Light assays and the plastic pegs in the crystal violet assays might explain the difference in bacterial retention on surfaces and the biofilm removal behaviour by

Listerine®. Although validation studies in this chapter showed the ability to reproducibly produce biofilms on the same substrata, it has been observed that the viable counts of the functional bacterial groups of the resuspended biofilms grown on glass slides were significantly lower than the viable counts of bacterial groups grown on HA substrata with respect to the surface area. It is proposed that the topography of glass substrata makes it less favourable for biofilm growth with the organisms tested, with a higher chance for biofilm integrity to be disrupted and removed on exposure to chemotherapeutic agents. Hence, a biofilm detachment activity was observed on mouthwash treated glass slides. The results presented in this chapter also showed a synergistic activity between two of the active ingredients of Listerine®, namely menthol and thymol. When tested on *in vitro* biofilm models, the paired essential oils showed a significant reduction in the viable counts, in some cases, similar to that of Listerine® mouthwash (Figs. 4.11, 4.12, 4.13 and 4.14). This synergy of the combinatorial essential oils was also observed against planktonic oral species in an FIC index study (Table 4.2). The revealed combinatorial activity of the active ingredients in Listerine® may provide a new insight into the action of an old and poorly understood formulation.

In summary, *in vitro* studies showed that the essential oil-based mouthwash, Listerine® was more effective against salivary-derived biofilms than the other tested mouthwashes. Its antibacterial efficacy was suggested to be related to its rapid kill ability rather than biofilm removal activity. It has also been indicated that the antimicrobial efficacy of Listerine® is mostly related to the synergistic activity of two of its active ingredients, the essential oils menthol and thymol.

CHAPTER 5

In vitro comparative evaluation of the anti-plaque effects of proprietary dentifrices

5.1 Introduction

Plaque has long been considered to be the main factor associated in the occurrence of oral diseases such as dental caries, gingivitis and periodontitis (Theilade 1986; Loesche 1992; Marsh 1994; Marsh and Martin 1999). The primary factor in achieving good oral health is the adequate routine control of dental plaque by both mechanical and chemical means. Self practiced procedures such as toothbrushing, alongside the use of mouthwashes and flossing are considered to be the most common methods in achieving good oral hygiene. In order to improve the efficacy of self-performed mechanical procedures such as tooth cleaning, antimicrobial molecules are commonly employed. A logical extension to the traditional role of toothpastes is to use them as carriers of anti-plaque agents (Svatun *et al.* 1990). A considerable number of antimicrobial agents have been evaluated for their use in dentifrices, of which very few have been adopted for use in commercial toothpastes that provide good oral hygiene. In this context, this chapter evaluates the potential use of toothpastes containing triclosan (Colgate Total®) and stannous fluoride with zinc lactate (Crest ProHealth®) to reduce *in vitro* bacterial viability. Although the antimicrobial efficacy of these oral dentifrices have been previously studied and comparatively evaluated using various approaches (Ledder *et al.* 2008), there is a lack in understanding of the antibacterial mechanism they produce and their effect on the plaque ecosystem.

5.2 Models/Approaches

Two *in vitro* models were adopted to give more useful insights on the antimicrobial efficacy of the proprietary toothpastes. The first was the hydroxyapatite disc model (HDM) which is a closed growth system model (Sections 2.12 and 4.1). The second model used the Sorbarod Biofilm Device (SBD) which is an example of an open growth system (continuous culture). This type of system yields relatively large amounts of bacterial biofilm mass and enables regular monitoring of bacterial functional groups dynamics through the analysis of spent culture fluid (perfusates) (Section 2.17). The rationale behind the use of the SBD, was to demonstrate the antibacterial efficacy of the tested dentifrices on a wider range of oral bacterial ecosystems, whereas the HDM is more specific in the detection of effects targeted on the supragingival plaque (McBain *et al.* 2005). Another significant advantage of an open system (continuous culture) over simple closed or multi-well plate models is that the mean growth rate can be altered by adjusting the rate of perfusion of medium (McBain *et al.* 2005). However, both systems provide information related to the comparative efficacy of the tested dentifrices against different functional groups of salivary microcosms; HDMs in short-term investigation and SBDs for longer durations.

5.3 Aims

The antimicrobial efficacy of an oral hygiene product is a representation of the efficacy of its active ingredient(s) and in some cases other components present in the formulation (Marsh 1992; McBain *et al.* 2010). The antimicrobial efficacy of triclosan and the paired active ingredients stannous fluoride with zinc lactate present in Colgate Total[®] and Crest ProHealth[®] respectively, have been described

in Chapter 3. The aim of this chapter therefore, was to compare and investigate the antimicrobial efficacy of the two dentifrices as a whole formulation using various *in vitro* model systems (HDM and SBD).

5.4 Methods

For the purpose of developing any product which benefits oral hygiene, it is essential to investigate the characteristics and the antibacterial efficacy of both actives and excipients before they are incorporated in the product. In this chapter, the commercially available toothpastes tested were Colgate Total® and Crest ProHealth®. Colgate Total® toothpaste uses triclosan as its active ingredient, whereas, Crest ProHealth® contains a combination of active ingredients. These are stannous fluoride and potentially also zinc lactate, although zinc lactate was claimed to be an inactive ingredient by the manufacturer on the product's label. The antimicrobial efficacies of both zinc lactate and stannous fluoride against oral species have been described in previous studies (Shah 1982; Tinanoff and Camosci 1984; Weber *et al.* 1995; Winkel *et al.* 2003; Marquis *et al.* 2005) and in Chapter 3 of this thesis. To better understand which ingredient(s) is/are responsible for Crest ProHealth®'s antimicrobial efficacy, it was important to investigate the antimicrobial efficacy of zinc lactate and stannous fluoride separately and in combination.

In order to evaluate the combinatorial activity of zinc lactate and stannous fluoride, a checkerboard microdilution method was chosen for the investigation of the antimicrobial efficacy of the active ingredients of Crest ProHealth®. This method is widely used for the detection of combinatorial activities between antimicrobial agents as previously discussed (Section 2.10).

5.5 Results

5.5.1 *In vitro* identification of claimed to be inactive ingredients

The concentrations of the tested antimicrobial agents were prepared to represent the concentrations present in the dentifrices as shown in Table 3.1. The interactions between the two antimicrobial agents were classified as synergistic, additive, or antagonistic on the basis of the fractional inhibitory concentration (FIC) index. The FIC index is the sum of the FICs of each of the antimicrobials where the FIC is defined as the MIC of each antimicrobial when used in combination divided by the MIC of the antimicrobial when used alone (Perea *et al.* 2002). The interaction was defined as synergistic if the FIC index was <1, additive if the FIC index was 1.0, sub-additive if the FIC index was between 1.0 and 2.0, indifferent if the FIC index was 2, and antagonistic if the FIC index >2 (Berenbaum 1978; Hall *et al.* 1983; Scott *et al.* 1999; Canton *et al.* 2005). In accordance to the FIC index values presented in Table 5.1 both antimicrobial agents (stannous fluoride and zinc lactate) have shown to possess a synergistic antimicrobial activity against all tested oral species.

5.5.2 *In vitro* evaluation of the antibacterial efficacy of two proprietary dentifrices (closed system model)

The hydroxyapatite disc model was one of the techniques that was used to evaluate and compare the antimicrobial efficacy of two oral dentifrices against various bacterial functional groups found in salivary microcosms (Section 2.12). The dentifrices that have been tested in this study included the following active ingredients *i)* Colgate Total®, contains 3mg.ml^{-1} of the active ingredient triclosan and *ii)* Crest ProHealth®, contains 4.5 and 10mg.ml^{-1}of stannous fluoride and zinc lactate respectively. It is important to note that the concentration used for each

tested dentifrice was 5%. The reasons behind using this concentration were; *i)* the high density of the suspension (reconstituted toothpastes) at higher concentrations led to an adhesion of the HA discs with the bottom of the wells of the culture plate causing a physical disruption of the grown biofilms, *ii)* concentrations greater than 5% were unable to form a uniform suspension after reconstitution giving false data interpretation and, *iii)* concentrations greater than 5% led to the formation of unsuspended toothpaste clumps adhering to the HA disc and physically disrupting the formed biofilm.

Fig. 5.1 illustrates the effects of 5 mins. exposure to Colgate and Crest ProHealth$^{®}$ dentifrices on the viable counts of functional bacterial groups of saliva-derived oral microcosms maintained on HA discs. The data presented in Fig. 5.1 show that both dentifrices were statistically significant ($p < 0.05$) in reducing the viable counts of total aerobic bacterial species compared to control. Viable counts of the total aerobic species achieved by exposure to both dentifrices separately was *ca.* 2 log_{10} cfu.mm^{-1} reduction, whereas the viable counts of both dentifrices were not significantly different ($p > 0.05$) when compared to each other. On 5 mins. exposure, both dentifrices were able to significantly reduce ($p < 0.05$) the viable counts of total facultative anaerobes by *ca.* 2.5 log_{10} cfu.mm^{-1} compared to control counts (Fig. 5.1). However, viable count reductions of the facultative anaerobes of both dentifrices were not statistically different when compared to each other (Fig. 5.1). Colgate Total$^{®}$ dentifrice was able to cause a reduction of *ca.* 2.5 log_{10}cfu.mm^{-1} to the viable counts of total Gram-negative anaerobes compared to the control counts, whilst Crest ProHealth$^{®}$ showed an *ca.* 2 log_{10} cfu.mm^{-1} reduction of the same functional bacterial group in comparison to control (Fig. 5.1). However, both dentifrices showed a statistically significant ($p < 0.05$) reduction of the total Gram-

Table 5.1: *In vitro* interactions between stannous fluoride and zinc lactate on various species of the oral microcosm indicated by the fractional inhibitory concentration index (FIC) method.

Organism	stannous fluoride (mg.ml^{-1})^a		zinc lactate (mg.ml^{-1})^b		stannous fluoride (mg.ml^{-1})^c		zinc lactate (mg.ml^{-1})^d		Checkerboard FIC index	Interpretation
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC		
S. oralis	0.5 0.5 (0.25)	ND	0.25	ND	1×10^{-3}	ND	0.016	ND	0.0644	S
S. mutans	0.25	ND	1.00 1.00 (0.5)	ND	1×10^{-3}	ND	0.016	ND	0.0195	S
P. oralis	0.25	ND	1.00	ND	1×10^{-3}	1.00	0.016	1.00	0.0195	S
S. sanguis	0.5	ND	0.25	ND	1×10^{-3}	1.00	0.016	1.00	0.0644	S
L. rhamnosus	1.00 1.00 (0.5)	ND	1.00	ND	9.8×10^{-4} (1.9×10^{-3})	ND	0.016	ND	0.0166	S
V. dispar	0.5	ND	1.00	ND	1×10^{-3}	0.5	0.016	0.016	0.0176	S
N. subflava	0.5	ND	0.25	1.00	1×10^{-3}	1×10^{-3}	0.016	1.00	0.0644	S
A. naeslundii	0.5 0.5 (0.25)	ND	1.00 1.00 (0.5)	ND	1×10^{-3} (1.9×10^{-3})	0.5	0.016	0.016	0.0176	S

^{a,b} ; MICs and MBCs are for the antimicrobials tested in separately, ^{c,d} ; MICs and MBCs are for the antimicrobial agents tested in combination, S ; Synergy, ND ; not detected.
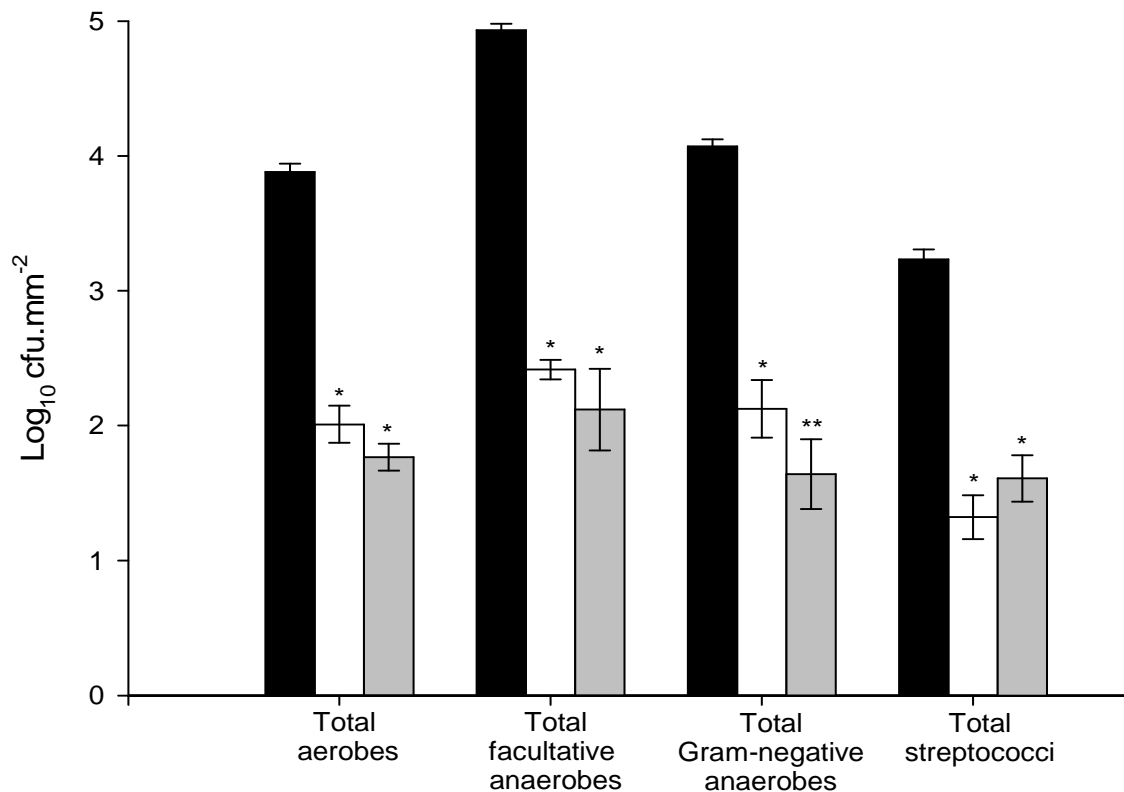
Figure 5.1: Effects of dentifrices on functional bacterial groups viable counts of saliva-derived oral microcosms maintained on HA discs after five mins. exposure. The solid black bars represent control counts (untreated microcosms), the open bars represent the treated biofilm with Crest ProHealth[®] dentifrice, while the solid gray bars represent the treated biofilm with Colgate Total[®] dentifrice . Data represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3). * above the bars represents a significant change with respect to control counts (p < 0.05). ** above the bars represents a significant change with respect to control counts and other dentifrice counts (p < 0.05).

negative anaerobes compared to the control. In addition, the data presented in Fig. 5.1 showed a significant reduction (p < 0.05) in the viable counts of total streptococci (*c.* 1.5 $\log_{10}$ cfu.mm^{-1}) after exposure by both dentifrices compared to control. However, there was no significant difference (p > 0.05) in the viable counts of the total streptococci of both dentifrices when compared to each other (Fig 5.1).

In summary, exposure to both Colgate Total[®] and Crest ProHealth[®] dentifrices separately resulted in significant reductions (p < 0.05) in viable counts of all bacterial functional groups of the tested oral microcosms in comparison to control. The reductions in the viable counts of all the tested salivary functional groups were not significantly different when compared between both toothpastes, an exception

of which were the viable counts of Gram-negative anaerobic species that have been reduced to a greater extent by exposure to Colgate Total[®] than Crest ProHealth[®].

5.5.3 *In vitro* evaluation of the antibacterial efficacy of two proprietary dentifrices (open system model)

The Sorbarod Biofilm Device (SBD) was selected as a second model for the evaluation and the comparison of the antimicrobial efficacy of Colgate Total[®] and Crest ProHealth[®] dentifrices against various bacterial functional groups present in the salivary microcosm (Section 2.16). Each SBD (n=4) was set up to run for a period of 9 days. On day six, two models were pulsed with the same dentifrice twice a day for a period of 3 days. The data in Fig. 5.2 show the results of culture-based enumeration of total aerobic bacteria for plaque microcosms before and after pulsing with the proprietary dentifrices.
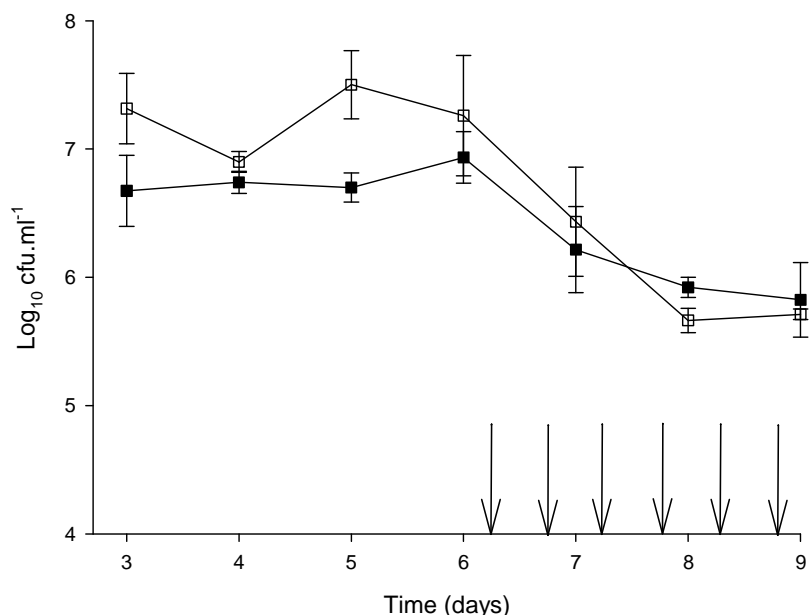
Figure 5.2: Viable counts of total aerobic bacteria in SBDs before and during pulsing with 5% w/v proprietary dentifrices. (–□–) mean of ecosystems pulsed with Colgate Total[®] (n=2); (–■–) mean of ecosystems pulsed with Crest ProHealth[®] (n=2); error bars represent standard errors of the mean between models (n=2). The vertical arrows show the time of dentifrices addition.

Dynamic stability of the characterised bacterial communities was attained in the sorbarod devices within *ca.* 3 days of inoculation. The mean values of the viable counts of total aerobic bacterial groups of the SBD models (n=2) pulsed with Colgate Total$^{®}$ show a reduction of 1.5 $\log_{10}$ cfu.mm^{-1}, whereas, the mean values of the viable counts of the same species derived from ecosystems pulsed with Crest ProHealth$^{®}$ show a lower reduction of 0.9 $\log_{10}$ cfu.mm^{-1} (Fig. 5.2). However, results did not show any statistical significance in the viable counts between models after pulsing with either dentifrice.

Figure 5.3 illustrates the viable counts of total anaerobic bacterial functional groups in SBD models before and during the addition of 5% Colgate Total$^{®}$ and Crest ProHealth$^{®}$ suspensions. The addition of Colgate Total$^{®}$ to the SBDs caused a slight reduction in viable counts of total anaerobic bacterial functional groups (*ca.* 1 $\log_{10}$ cfu.mm^{-1}) as shown in Fig. 5.3.
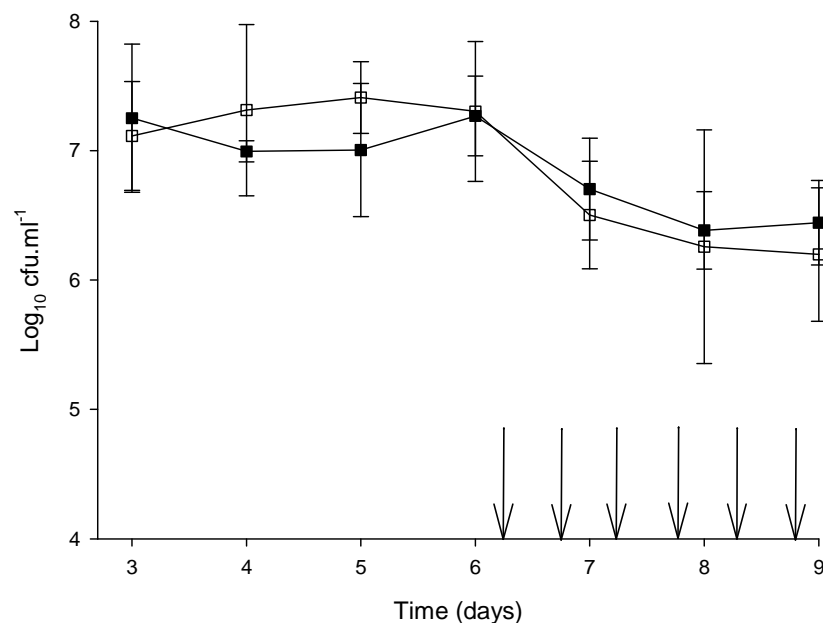
Figure 5.3: Viable counts of total anaerobic bacterial groups in SBDs before and during pulsing with 5% w/v proprietary dentifrices. (—□—) mean of ecosystems pulsed with Colgate Total$^{®}$ (n=2); (—■—) mean of ecosystems pulsed with Crest ProHealth$^{®}$ (n=2); error bars represent standard errors of the mean between models (n=2). The vertical arrows show the time of dentifrices addition.

Pulsing with Crest ProHealth® dentifrice suspension showed a reduction (*ca.* 0.9 $\log_{10}$ cfu.mm^{-1}) in the viable counts of anaerobic species of perfusate samples from the represented SBDs (Fig. 5.3). However, the mean values of the reduction of viable counts of anaerobic species were not statistically different between the models after pulsing with the tested dentifrices.

Figure 5.4 shows the data obtained from culture-based enumeration of total Gram-negative anaerobic bacteria for plaque microcosms before and after pulsing with the proprietary dentifrices. Pulsing with Colgate Total® suspension showed a reduction in the viable counts of total Gram-negative anaerobic bacterial groups in the represented models, where this reduction reached *ca.* 2 $\log_{10}$ cfu.mm^{-1}.
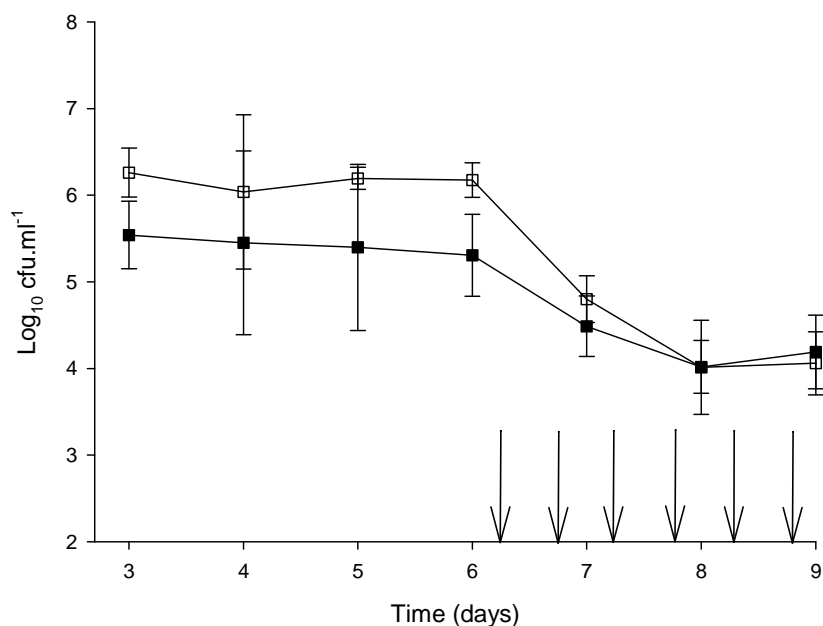
Figure 5.4: Viable counts of total Gram-negative anaerobic bacterial groups in SBDs before and during pulsing with 5% w/v proprietary dentifrices. (—□—) mean of ecosystems pulsed with Colgate Total® (n=2); (—■—) mean of ecosystems pulsed with Crest ProHealth® (n=2); error bars represent standard errors of the mean between models (n=2). The vertical arrows show the time of dentifrices addition.

A lower reduction in the viable counts (*ca.* 1.1 $\log_{10}$ cfu.mm^{-1}) of Gram-negative species was observed after pulsing the SBDs with Crest ProHealth® dentifrice suspension as shown in Fig 5.4. Despite the high difference in the reductions of the viable counts, the mean values of the reduction of viable counts of Gram-negative species were not statistically different between the models after pulsing with the tested dentifrices.
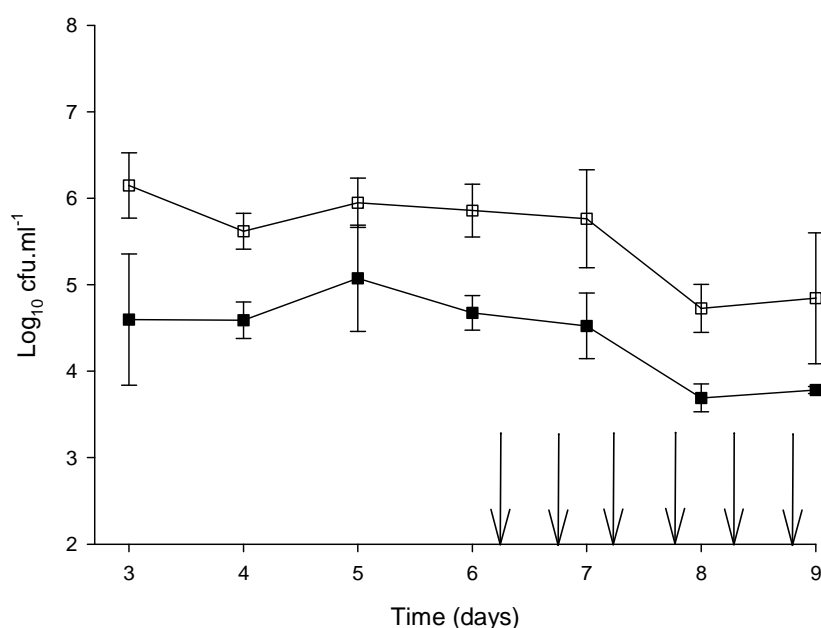
Figure 5.5: Viable counts of total streptococci bacterial groups in SBDs before and during pulsing with 5% w/v proprietary dentifrices. (—□—) mean of ecosystems pulsed with Colgate Total® (n=2); (—■—) mean of ecosystems pulsed with Crest ProHealth® (n=2); error bars represent standard errors of the mean between models (n=2). The vertical arrows show the time of dentifrices addition.

The effects of the addition of proprietary dentifrices on the reduction in viable counts of total streptococci in dental microcosms have been illustrated in Fig. 5.5. A minor transient reduction in the counts of streptococci (*ca.* 0.7 $\log_{10}$ cfu.mm^{-1}) was noted after pulsing models with Colgate Total® toothpaste suspensions. Pulsing with Crest ProHealth® also had a minor effect in reducing the viable counts of streptococci species in SBDs. This reduction was also estimated to be *ca.* 0.7 $\log_{10}$ cfu.mm^{-1} (Fig. 5.5).

In general, the SBDs that have been dosed with Colgate Total[®] and Crest ProHealth[®] respectively, showed a similar reduction in the viable counts of streptococci bacterial groups.

5.6 Discussion

The association between plaque and oral disease has been well established and comprehensively studied over the years (Löe *et al.* 1967; Tanner *et al.* 1996; Tanner *et al.* 1998)**.** The necessity to control plaque by the adjunct use of oral hygiene products containing antimicrobial agents with mechanical control has also been described and studied (Axelsson and Lindhe 1987; DePaola and Daniel 1989; McBain *et al.* 2003; Rawlinson *et al.* 2008). A variety of over-the-counter oral hygiene preparations with various antimicrobial agents have been introduced to the public. Many of these products claim to be beneficial for the control of oral hygiene and the prevention of oral disease such as dental caries, gingivitis and periodontitis. Various models and approaches, both *in vitro* and clinical trials, have been used to compare the anti-plaque efficacy of various oral preparations such as dentifrices and mouthwashes (Mankodi *et al.* 1987; Jenkins *et al.* 1993; McBain *et al.* 2003). The current chapter investigated the antimicrobial efficacy of two different dentifrices containing previously studied antimicrobial agents (Chapter 3). The first dentifrice contained the antimicrobial agent triclosan (Colgate Total[®]), whilst the second dentifrice contained the paired antimicrobial agents stannous fluoride and zinc lactate (Crest ProHealth[®]). Various models have been used to compare the antibacterial effects of Colgate Total[®] and Crest ProHealth[®] as whole formulations and active ingredients on *in vitro* oral biofilms (Ledder *et al.* 2008; McBain *et al.* 2010). In previous studies modified drip flow biofilm reactors and

Multiple Sorbarod Devices were used to establish salivary biofilm microcosms to evaluate the antimicrobial effects of these proprietary toothpastes (Ledder *et al.* 2008). The experimental models adopted in this chapter have been used to compare the micro-ecological effects of single (HDM) and multiple dosing (SBD) of the commercial toothpaste.

The hydroxyapatite disc model has been previously used to investigate the antimicrobial efficacy of oral hygiene products (Shapiro *et al.* 2002). In the current chapter, the ability of Colgate Total® and Crest ProHealth® to reduce viable counts of mixed microbial populations was examined using the HDM. *In vitro* investigations using the HDMs showed that both tested dentifrices were equally effective against most salivary bacterial functional groups. However, a higher reduction in the Gram-negative bacterial functional groups was noted after exposure to Colgate Total®.

The continuous culture SBD was the second type of model that was used in the current chapter. The use of continuous culture models to evaluate the efficacy of oral hygiene products has previously been investigated (Pratten *et al.* 1998; McBain *et al.* 2003). However, the SBD has not been used to evaluate the effectiveness of dental hygiene products namely, toothpastes against oral microcosms. The use of the continuous culture models such as the SBD may be a useful tool in studying microcosms that simulate and resemble the subgingival plaque. Since *in vitro* dosing regimes do not necessarily reproduce the dynamics and variability of actual human use, four *in vitro* models were set up to reduce inter-models variability and give an indication of consortial responses to dentifrice pulsing.

Bacteriological data generated after multiple dosing of the SBD models with the dentifrices suspensions showed that, as with some HDM biofilm functional groups, both dentifrices were equally effective in the reduction of all tested functional groups. The inhibitory activity of Colgate Total® toothpaste against Gram-negative anaerobic functional group in SBDs was particularly marked, however statistically insignificant compared to its counterpart Crest ProHealth®. Colgate Total® toothpaste's inhibitory activity against Gram-negative species, as in SBD models, was also significantly reduced in the HDM plaques with respect to the amplitude of viability reductions of both models. These reductions of the Gram-negative groups supported the results shown for the same species but investigated by other models in a previous study (Ledder *et al.* 2008). However, the substrata, the dosing regimen and the concentrations of the dentifrices tested were the main differences between the previous and the current study. The main advantage in this study over the previous study was using lower concentrations of the dentifrices (5%) to form a uniform dentifrice suspension and prevent clump formation which could physically disrupt the formed biofilms. Another advantage was dosing the SBD models with the dentifrices twice daily to simulate the daily routine use of oral hygiene products. In the current study however, Crest ProHealth® was observed to be as successful as Colgate Total® in reducing the viable counts of all bacterial functional groups in both single and multiple dosing approaches, with an exception of Gram-negative groups grown in HDM plaques, where the reduction in viable counts was greater after exposure to Colgate Total®. Since most of the known periodontal pathogens are Gram-negative anaerobes (Socransky and Haffajee 2005), making this bacterial functional group a key target for oral hygiene products. The ability of Colgate Total® toothpaste to markedly

reduce this bacterial functional group after exposure in the HDM, with respect to oral health, highly suggest its clinical efficacy against the progression of periodontal diseases as previously proven by (Rosling *et al.* 1997; Cullinan *et al.* 2003).

In general, both Colgate Total® and Crest ProHealth® toothpastes caused a reduction in bacterial diversity in both tested bacterial plaques. However, this diversity was more reduced in cases of exposure with Colgate Total®. It can also be indicated that the comparative efficiencies of both tested formulations were broadly congruent with previous studies using active ingredients alone (Gilbert *et al.* 2003; McBain *et al.* 2010).

CHAPTER 6

General discussion

Throughout life, the human body is in continual contact with a variety of microorganisms, the majority of which are eubacteria. An important outcome of the co-evolution between humans and bacteria is the development of complex, intimate and stable relationships (Rasiah *et al.* 2005). Such host-bacterial symbiotic relationships may be *i)* commensal, in which microorganism benefit from the substrate and substratum while the host neither benefit nor harmed, *ii)* mutual, in which reciprocal benefits occur to both microorganism and host, or *iii)* pathogenic in which microorganisms benefit whilst the host is harmed. Similar to other sites in the body such as the large intestine and the skin, the oral cavity has a relatively stable characteristic composition of bacterial flora that co-exists with the host cells (Marsh 2003). The physiological properties of different parts in the oral cavity provide a suitable environment for the growth and colonisation of a wide range of bacterial species. Distinct areas of the mouth include soft mucosal surfaces such as the tongue, cheeks, lips and the hard non-shedding surfaces of teeth. Microbial biofilms growing on the non-shedding surfaces of the teeth are known as supragingival plaque, whilst the gingival crevice provides a nutritionally unique habitat for the growth of a taxonomically distinct microbiota, known as subgingival plaque (Slots 1977). Breakdowns in the microbial homeostasis of dental plaque may change the mutual host-bacterial relationship into a pathogenic relationship (Marsh 1989; Newman 1990). The outcome of the overall pathogenic effect of dental plaque is the emergence of major oral diseases such as dental

caries, gingivitis and periodontitis. Importantly however, periodontal disease may expose the blood stream to opportunistic pathogens that maybe the causative agents of systemic diseases such as endocarditis, meningitis and septicaemia (Lewin and Hughes 1966; Pollack and Mogtader 1984; Amsel and Mouljn 1996; Baraldès *et al.* 2000). Hence, the development of effective hygienic products which effectively control bacterial growth in the mouth is of great interest (Gordon *et al.* 1985; Gilbert *et al.* 2003; McBain *et al.* 2003; Ledder *et al.* 2009). In this context, attempts to control plaque accumulation or prevent its harmful effects continue to be primary means by which dental plaque-related diseases are controlled or prevented (Daniel *et al.* 1990; Giertsen 2003; Ouhayoun 2003). Since they serve as a valuable complement to mechanical removal of plaque, various antimicrobial agents have been deployed with many different oral hygiene control formulations such as toothpastes, mouthwashes, sprays and gels (Marsh 1992; McBain *et al.* 2010). A considerable number of antimicrobial agents have been evaluated for their use in oral formulations, of which a small proportion has been adopted for use in commercial products. For example, various essential oil preparations, antiseptic bisbiguanides, quaternary ammonium compounds, oxygenating agents, fluorinated compounds, metal salts, phenolic compounds and surfactants have been tested and developed in commercial mouthwashes and dentifrices (Horowitz 1971; Wennstorm and Lindhe 1979; Gordon *et al.* 1985; Stephen *et al.* 1990; Jenkins *et al.* 1991; McBain *et al.* 2003). In spite of the long and extensive use of oral antimicrobial agents, there remains a lack of understanding of their mechanisms of action on the bacterial cell at both effective and sub-lethal concentrations (sub-MIC levels) and on biofilm communities (plaque). Therefore, investigations in this doctoral dissertation aimed to better

understand the effects of selected oral antimicrobial agents on salivary microcosms and axenic species. In this respect, the outcome of effective and sub-lethal concentrations of selected antimicrobial agents (triclosan, stannous fluoride, sodium lauryl sulphate and zinc lactate) on cellular respiration/fermentation of *N. subflava, S. oralis* and *E. coli* species was investigated since acidic fermentation products play a major factor in enamel erosion. Antimicrobial agents which divert cellular metabolism from respiration towards fermentation may result in greater acidity in the oral cavity and conversely, acidogenesis and thus cariogenesis may be decreased by agents which encourage respiratory metabolism. The use of some products may therefore lead to comparatively greater enamel erosion due to changes in cellular respiration/fermentation. The effects of dentifrices containing the above selected antimicrobial agents on plaque accumulation, inactivation and taxonomical composition were therefore investigated.

Since oral care products are deployed as complex formulations containing several ingredients (actives and excipients) that enhance the overall effectiveness of a formulation, this thesis aimed to provide further knowledge regarding the contribution of selected ingredients on the total antimicrobial efficacy of a complex formulation. The antibacterial efficacies of selected antimicrobial agents and formulations were investigated using appropriate *in vitro* model systems. Closed systems (the hydroxyapatite disc model) and open/continuous flow system (the Sorbarod Biofilm device) were used to reproduce oral bacterial ecosystems that simulate distinct microbial habitats in the oral cavity whilst microdilution methods (microtiter plate assays) were used to grow axenic bacterial species. Bacterial culture techniques using selective bacteriological media were used to measure

viable counts of specific bacterial functional groups after exposure to various oral antimicrobial agents.

Data presented in Chapter 3 make the observation that triclosan and sodium lauryl sulphate are responsible for the uncoupling of the proton motive force from the phosphorylation of ADP to ATP in the tested microorganisms. This phenomenon occurred in both long- and short-term experiments. Data generated also suggested that long-term exposure of facultative anaerobic species (*S. oralis* and *E. coli*) to the sub-MIC levels of stannous fluoride cause shifts in metabolic activity towards fermentation. Such changes could lead to an adverse impact on the oral hygiene by providing reduced environmental conditions. This in turn could lead to the increase in acidogenic bacterial species population and consequently lead to an increase in harmful fermentation product (lactic acid) which could cause enamel erosion and caries (Bradshaw and Marsh 1988; Marsh and Martin 1999).

Data in Chapter 4 suggest that Listerine® mouthwash caused greater reductions in total counts of various bacterial functional groups (total aerobes, total facultative anaerobes, total Gram-negative anaerobes and total streptococci species) after one min. exposure compared to the other tested mouthwashes (Neutrafluor®, Plax®overnight®, Periogard® and Peroxyl®) and the control. The results also show that active ingredients of Listerine® mouthwash (thymol, menthol, methyl salicylate and eucalyptol) produced a modest reduction in the viable counts of bacterial functional groups when investigated separately rather than in combination. The antimicrobial efficacy of Listerine® mouthwash was more attributed to the novel observation of synergistic antimicrobial activity of its active ingredients menthol and thymol, rather than the activity of the active ingredients singly (Chapter 4). Microscopy studies presented in Chapter 4 suggest that Listerine® mouthwash has

effective biofilm penetration and/or bacterial detachment activity when added to cultures grown on glass slides. This has been previously reported by Ouhayoun (2003). By comparison, *in vitro* studies using plastic pegs as the substrata showed no significant difference between treated and untreated biofilms.

Data presented in Chapter 5 described a synergy between the active ingredient stannous fluoride and zinc lactate in Crest ProHealth® dentifrice. Bacteriological data generated from two different *in vitro* biofilm models (the hydroxyapatite disc model and the Sorbarod Biofilm device) suggest that the dentifrice Colgate Total® was more effective than Crest ProHealth® dentifrice in the reduction of total Gram-negative bacteria functional groups in the HDM model. This is in agreement with previous studies (Ledder *et al.* 2008; Chapter 5). Results obtained by dosing plaque ecosystems in the SBDs with 5% suspensions of dentifrice indicate a similar decrease in the viable counts of all tested bacterial functional groups and suggesting a similar antimicrobial activity of both dentifrices (Chapter 5).

Observations presented in this doctoral dissertation provide a better understanding of properties of a variety of actives and may contribute to the development of oral formulations with optimised antimicrobial properties against adventitious pathogens present in the oral cavity and help in reducing the incidence of oral diseases and potentially related systemic interface.

With regards to the uncoupling activity demonstrated in this thesis (Chapter 3), the development and the regular use of oral formulations containing uncoupling agents may affect plaque metabolism and accumulation. This could be a result of the inability of bacterial cells to grow in the presence of the uncoupling agents. However, such exposed cells may continue to respire and thus utilise available

substrates present in the oral cavity. Consequently, this activity could result in plaque disruption.

The synergistic antimicrobial activity of the essential oils thymol and menthol which caused antibacterial effects of comparative magnitude to the whole formulation, as discovered in this study, could also be exploited by reducing or excluding the use of other ingredients in the formulation. This could contribute in reducing the overall side effects and increase the toxic margin of the whole formulation. However, physiochemical compatibility issues between the oils could rise when deployed alone which need further investigation.

The ability of sub-lethal levels of some antimicrobial agent (stannous fluoride and zinc lactate) to shift cellular metabolic activity towards fermentative pathways suggests that the effects of their long-term use should be investigated. Investigating the pH and the taxonomical composition of dosed plaque communities, could help provide further knowledge on the overall metabolic behaviour of plaque communities and any potential shifts in caries and periodontitis associated pathogens.

References

Aas, J., B. J. Paster, L. N. Stokes, I. Olsen and F. E. Dewhirst (2005). "Defining the normal bacterial flora of the oral cavity." *American Society for Microbiology* 43(11): 5721-5732.

American Dental Association (1986). "Guidelines for acceptance of chemotherapeutic products for the control of supragingival dental plaque and gingivitis. Council on Dental Therapeutics." *Journal of the American Dental Association* 112: 529–532.

Adams, D. and M. Addy (1994). "Mouthrinses." *Advanced Dental Research* 8(2): 291-301.

Addy, M., J. Moran and A. Griffiths (1985). "Extrinsic tooth discolouration by metals and chlorhexidine." *British Dental Journal* 159: 331-334.

Addy, M., M. A. Slayne and W. G. Wade (1992). "The formationn and control of dental plaque- an overview." *Journal of Applied Bacteriology* 73: 269-278.

Al-Bakri, A. G., P. Gilbert and D. G. Allison (2004). "Immigration and emigration of Burkholderia cepacia and *Pseudomonas aeruginosa* between and within mixed biofilm communities." *Journal of Applied Microbiology* 96(3): 455-463.

Al-Hashimi, I. and M. J. Levine (1989). "Characterization of *in vivo* saliva-derived enamel pellicle." *Archives in Oral Biology* 34: 289–295.

Allison, D. G. (2003). "The biofilm matrix." *Biofouling* 19: 139–150.

Amano, A. (2010). "Bacterial adhesins to host components in periodontitis." *Periodontology 2000* 52: 12-37.

Amano, A., A. Sharma, H. T. Sojar, H. K. Kuramitsu and R. J. Genco (1994). "Effects of temperature stress on expression of fimbriae and superoxide dismutase by *Porphyromonas gingivalis*." *Infection and Immunity* 62(10): 4682-4685.

Amsel, B. J. and A. C. Mouljn (1996). "Nonfebrile mitral valve endocarditis due to *Neisseria subflava*." *CHEST* 109: 280-282.

Andres, C. J., J. C. Shaeffer and A. S. Windeler (1974). "Comparison of antibacterial properties of stannous fluoride and sodium fluoride mouthwashes." *Journal of Dental Research* 53: 457-460.

Arro, L. and C.-R. Salenstedt (1973). "Evaluation of toxicity of some quaternary ammonium compounds." *Journal of Biological Standardization* 1: 11-22.

Axelsson, P. and J. Lindhe (1987). "Efficacy of mouthrinses in inhibiting dental plaque and gingivitis in man." *Journal of Clinical Periodontology* 14(4): 205-212.

Babich, H. and G. Stotzky (1978). "Toxicity of zinc to fungi, bacteria, and coliphages: influence of chloride ions." *Applied Environmental Microbiology* 36(6): 906-914.

Babson, L. and S. R. Babson (1973). "Kinetic colorimetric measurement of serum lactate dehydrogenase activity." *Clinical Chemistry* 19(7): 766-769.

Baehni, P. C. and Y. Takeuchi (2003). "Anti-plaque agents in the prevention of biofilm-associated oral diseases." *Oral Diseases.* 9: 23–29.

Bagg, J., M. P. Sweeny, K. Harvey Wood and A. Wiggins (1995). "Possible role of *Staphylococcus aureus* in severe oral mucositis among elderly dehydrated patients." *Microbial Ecology In Health And Disease* 8: 51-56.

Baker, Z., R. W. Harrison and B. F. Miller (1940). "Action of synthetic detergents on the metabolism of bacteria." *Science* 91: 249-271.

Baraldès, M. A., P. Domingo and J. Barrio (2000). "Meningitis due to *Neisseria subflava*: case report and review." *Clinical Infectious Diseases* 303: 615-617.

Barros, S., D. Barrow, S. Offenbacher, S. Wirojchanasak, F. Panagakos and W. DeVizio (2010). "Triclosan inhibition of acute and chronic inflammatory gene pathways." *Journal of Clinical Periodontology* 37(5): 412–418.

Beerns, H. (1990). "An elective and selective isolation medium for *Bifidobacterium* spp. ." *Letters in Applied Microbiology* 11: 155-157.

Berenbaum, M. C. (1978). "A method for testing for synergy with any number of agents." *Journal of Infectious Diseases* 137(2): 122-130.

Bhupathiraju, V. K., D. Landfear and L. Alvarez-Cohen (1999). "Application of a tetrazolium dye as an indicator of viability in anaerobic bacteria." *Journal of Microbiological Methods* 37: 231–243.

Bibby, B. G. and M. Van Kerstern (1940). "The effect of fluorine on mouth bacteria." *Journal of Dental Research* 19: 391-402.

Bibby, B. G., H. A. Zander, M. McKelleget and B. Labunsky (1946). "Preliminary reports on the effect on dental caries of the use of sodium fluoride in a prophylactic cleaning mixture and in a mouthwash." *Journal of Dental Research* 25: 207-211.

Bochner, B. R. and M. A. Savageau (1976). "Generalized indicator plate for genetic, metabolic, and taxonomic studies with microorganisms." *Applied Environmental Microbiology* 33(2): 434-444.

Boudewijns, M., K. Magerman, A. Mewis, V. Peeters, J. L. Rummens, R. Cartuyvels, J. Verhaegen, G. Debrock, W. E. Peetermans and P. Donkersloot (2003). "*Rothia dentocariosa*, endocarditis and mycotic aneurysms: case report and review of the literature." *Clinical Microbiology and Infection* 9: 222-229.

Bowden, G. H., J. M. Hardie and G. L. Slack (1975). "Microbial variations in approximal dental plaque." *Caries Research* 9: 253–277.

Bowden, G. H. and Y. H. Li (1994). "Characteristics of accumuiation of oral gram-positive bacteria on mucin-conditioned glass surfaces in a model system." *Oral Microbiology and Immunology* 9: 1-11.

Boyar, R. M. and G. H. Bowden (1985). "The microflora associated with the progression of incipient lesions in teeth of children living in a water fluoridated area." *Caries Research* 19(4): 298-306.

Boyd, R. D., D. Cole, D. Rowe, J. Verran, S. J. Coultas, A. J. Paul, R. H. West and D. T. Goddard (2000). "Surface characterization of glass and poly(methyl methacrylate) soiled with a mixture of fat, oil, and starch." *Journal of Adhesion Science and Technology* 14: 1195-1207.

Bradshaw, D. J. and P. D. Marsh (1988). "Analysis of ph-driven disruption of oral microbial communities *in vitro*." *Caries Research* 32: 456-462.

Bradshaw, D. J., P. D. Marsh, C. Allison and K. Schilling (1996). "Effect of oxygen, inoculum composition and flow rate on development of mixed-culture oral biofilms " *Microbiology* 142(623-629).

Bradshaw, D. J., P. D. Marsh, G. K. Watson and C. Allison (1998). "Role of *Fusobacterium nucleatum* and coaggregation in anaerobe survival in planktonic and biofilm oral microbial communities during aeration." *Infection and Immunity* 66: 4729–4732.

Brailsford, S. R., B. Shah, D. Simons and I. I (2001). "The predominant aciduric microflora of root-caries lesions." *Journal of Dental Research* 80(9): 1828-1833.

Bramanti, T. E. and S. C. Holt (1990). "Iron-regulated outer membrane proteins in the periodontopathic bacterium, *Bacteroides gingivalis*." *Biochemical and Biophysical Research Communications* 166: 1146-1154.

Bridson E.Y, B. A. (1970). Methods In Microbiology. London, Academic Press.

Brock, T. D., M. Madigan, J. Martinko and J. Parker (1994). Brock Biology of Microorganisms. 7 ed. Prentice Hall.

Brokstad, K. A., V. Bakken, E. N. Vasstrand and H. B. Jensen (1990). "Diisopropylfluorophosphate-binding proteins in the outer membrane of *Fusobacterium nucleatum*: strain variations." *Federation of European Microbiological Societies* 66: 235-238.

Busscher, H. J. and H. C. van der Mei (1997). "Physico-chemical interactions in initial microbial adhesion and relevance for biofilm formation." *Advances in Dental Research* 11: 24–32.

Canton, C., J. Peman and M. Gobernado (2005). "Synergistic activities of fluconazole and voriconazole with terbinafine against four candida species determined by checkerboard, time-kill, and etest methods." *Antimicrobial Agents and Chemotherapy* 49(4): 1593-1596.

Carlsson, J., H. Grahnen, G. Jonsson and S. Wikner (1970). "Early establishment of *Streptococcus salivarius* in the mouths of infants." *Journal of Dental Research* 49(2): 415-418.

Cassels, F. J., C. V. Hughes and J. L. Nauss (1995). "Adhesin receptors of human oral bacteria and modeling of putative adhesin-binding domains." *Journal of Industrial Microbiolology* 15: 176–185.

Chan, E. C. S. and R. McLaughlin (1999). "Taxonomy and virulence of oral spirochetes." *Oral Microbiology and Immunology 2000* 15: 1-9.

Chander, S., C. C. Chiao and B. W. Fuerstenau (1982). "Transformation of calcium fluoride for caries prevention." *Journal of Dental Research* 61: 403-407.

Chawner, J. A. and P. Gilbert (1989a). "A comparative study of the bactericidal and growth inhibitory activities of the bisbiguanides alexidine and chlorhexidine." *Journal of Applied Bacteriology* 66: 243–252.

Chawner, J. A. and P. Gilbert (1989b). "Interaction of the bisbiguanides chlorhexidine and alexidine with phospholipid vesicles: evidence for separate modes of action." *Journal of Applied Bacteriology* 66: 253–258.

Chawner, J. A. and P. Gilbert (1989c). "Adsorption of the bisbiguanides alexidine and chlorhexidine to *Escherichia coli* cells and to isolated membrane components." *International Journal of Pharmaceutics* 55: 209–215.

Choi, B. K., B. J. Paster, F. E. Dewhirst and U. B. Goble (1993). "Diversity of cultivable and uncultivable oral spirochetes from a patient with severe destructive periodontitis." *American Society for Microbiology* 62(5): 1889-1895.

Claesson, R., M.-B. Edlund, S. Persson and J. Carlsson (1990). "Production of volatile sulfur compounds by various *Fusobacterium* species." *Oral Microbiology and Immunology* 5: 137-142.

Collins, M. D. (1982). "Reclassification of *Bacterionema matruchotii* (Mendel) in the genus Corynebacterium , as Corynebacterium matruchotii comb.nov." *ZENTRALBL. BAKTERIOL., MIKROBIOL. HYG., I ABT. C* 3(3): 364-367.

Crociani, F., B. Biavati, A. Alessandrini, C. Chiarini and V. Scardovi (1996). "*Bifidobacterium inopinatum* sp. nov. and *Bifidobacterium denticolens* sp. nov., two new species isolated from human dental caries." *International Journal of Systematic Bacteriology* 46(2): 564-571.

Cullinan, M. P., B. Westerman, S. M. Hamlet, J. E. Palmer, M. J. Faddy, G. J. Seymour (2003). "The effect of a triclosan-containing dentifrice on the progression of periodontal disease in an adult population." *Journal of Clinical Periodontology* 30(1): 414-419.

Cummins, D., G. K. Watson, P. D. Marsh and D. J. Bradshaw (1993). "The effects of triclosan and zinc citrate, alone and in combination, on a community of oral bacteria grown *in vitro*." *Journal of Dental Research* 72(1): 25-30.

Cunliffe, D., C. A. Smart, C. Alexander and E. N. Vulfson (1999). "Bacterial adhesion at synthetic surfaces." *Applied Environmental Microbiology* 65(11): 4995-5002.

Daniel, C., F. Timothy, L. G. DePaola, M. G. DePaola and E. Glenn (1990). "Comparative effects of 2 chemotherapeutic mouthrinses on the development of supragingival dental plaque and gingivitis." *Journal of Clinical Periodontology* 17(575-579).

Darras-Joly, C., O. Lortholary, J. Mainardi, J. Etienne, L. Guillevin and L. Acar (1997). "*Haemophilus endocarditis*: report of 42 cases in adults and review. *Haemophilus Endocarditis* Study Group." *Clinical Infectious Diseases* 24(6): 1087-1094.

Davies, A. (1973). "The mode of action of chlorhexidine." *Journal of Periodontology* 8: 68–75.

de Almeida, P., A.-M. Gregio, M. Machado, A. de Lima and L. Azevedo (2008). "Saliva composition and functions: a comprehensive review." *The Journal of Contemporary Dental Practice* 9(3): 1-11.

De Boever, D. H. and W. J. Loesche (1995). "Assessing the contribution of anaerobic microflora of the tongue to oral malodor." *Journal of American Dental Association* 126: 1384-1392.

de Jong, M. H. and J. S. Van der Hoeven (1987). "The growth of oral bacteria on saliva." *Journal of Dental Research* 66(2): 498-505.

de Leeuw, N. H. (2004). "Resisting the onset of hydroxyapatite dissolution through the incorporation of fluoride." *Journal of Physical Chemistry* 108: 1809-1811.

de Stoppelaar, J., J. Van Houte and B. Dirks (1970). "The effect of carbohydrate restriction on the presence of *Streptococcus mutans*, *Streptococcus sanguis* and iodophilic polysaccharide-producing bacteria in human dental plaque." *Caries Research* 4: 114-123.

DePaola, L. G. and C. Daniel (1989). "Chemotherapeutic inhibition of supragingival dental plaque and gingivitis development." *Journal of Clinical Periodontology* 16: 311-315.

Dewhirst, F. E., B. J. Paster, N. Tzellas, B. Coleman, Downes, D. A. Spratt and W. G. Wade (2001). "Characterization of novel human oral isolates and cloned 16S rDNA sequences that fall in the family Coriobacteriaceae: description of olsenella gen. nov., reclassi cation of *Lactobacillus uli* as *Olsenella uli* comb. nov. and description of *Olsenella profusa* sp. nov." *International Journal of Systematic and Evolutionary Microbiology* 51: 1797-1804.

Donlan, R. M. and J. W. Costerton (2002). "biofilms: survival mechanisms of clinically relevant microorganisms." *Clinical Microbiology Reviews* 15(2): 167-193.

Doref, G. J., J. Jackson and M. W. Rytel (1974). "Infections with *Eikenella corrodens.* A newly recognized human pathogen." *Annals of Internal Medicine* 80(3): 305-309.

Dorthu, M. P., S. Remy, M.-R. Michel-Wolwertz, D. Breyer and M.-C. Beckers (1992). "Biochemical, genetic and molecular characterization of new respiratory-deficient mutants in *Chlamydomonas reinhardtii*." *Plant Molecular Biology* 18: 759-772.

Dzink, J. L., S. Socransky and A. Haffajee (1988). "The predominant cultivable microbiota of active and inactive lesions of destructive periodontal diseases." *Journal of Clinical Periodontology* 15: 316-323.

Dzink, J. L., A. C. R. Tanner, A. Haffajee and S. Socransky (1985). "Gram-negative species associated with active destructive periodontal lesions." *Journal of Clinical Periodontology* 12: 648-659.

Edgar, W. M. (1976). "The role of saliva in the control of ph changes in human dental plaque." *Caries Research* 10(4): 241-254.

Eiken, M. (1958). "Studies on an anaerobic, rod-shaped, gram-negative microorganism: *Bacteroides corrodens* n. sp." *Acta Pathologica Microbiologica Scandinavica* 43(4): 440-416.

Farrell, S., R. A. Baker, M. Somogyi-Mann, J. J. Witt and R. W. Gerlach (2006). "Oral malodor reduction by a combination of chemotherapeutical and mechanical treatments." *Clinical Oral Investigations* 10: 157–163.

Fedi, P. F. and W. J. Killoy (1992). "Temperature differences at periodontal sites in health and disease." *Journal of Periodontology* 63: 24-27.

Filip, C., G. Fletcher, J. L and C. F. Earhart (1973). "Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium-lauryl sarcosinate." *Journal of Bacteriology* 115(3): 717-722.

Fine, D. H., D. Furgang and M. L. Barnett (2001). "Comparative antimicrobial activities of antiseptic mouthrinses against isogenic planktonic and biofilm forms of *Actinobacillus actinomycetemcomitans*." *Journal of Clinical Periodontology* 28: 697-700.

Fouad, A., J. Barry, Q. Zhu, R. Carver, C. Caimano, M. Clawson, K. Hazlett and J. D. Radolf (2002). "PCR-based identification of bacteria associated with endodontic infections." *American Society for Microbiology* 40(9): 3223-3231.

Foulkes, D. M. (1973). "Some toxicological observations on chlorhexidine." *Journal of periodontal Research* 12(8): 55-57.

Fukui, M. and S. Takii (1989). "Reduction of tetrazolium salts by sulfate-reducing bacteria." *Federation of European Microbiological Societies* 62(13-20).

Gallagher, I. H. and T. W. Cutress (1977). "The effect of trace elements on the growth and fermentation by oral streptococci and actinomyces." *Archives of Oral Biology* 22: 555-562.

Gharbia, S. E. and H. N. Shah (1988). "Glucose utilization and growth responses to protein hydrolysates by *Fusobacterium* species. ." *Current Microbiology* 17: 229-234.

Gharbia, S. E., H. N. Shah and S. G. Welch (1989). "The influence of peptides on the uptake of amino acids in *Fusobacterium*; predicted interactions with *Porphyromonas gingivalis.*" *Current Microbiology* 19: 231-235.

Gibbons, R. J. (1984). "Adherent interactions which may affect microbial ecology in the mouth." *Journal of Dental Research* 63(3): 378-385.

Gibbons, R. J. and J. van Houte (1970). "Selective bacterial adherence to oral epithelial surfaces and its role as an ecological determinant." *American Society for Microbiology* 3(4): 567-573.

Giertsen, E. (2003). "Effects of mouthrinses with triclosan, zinc ions, copolymer, and sodium lauryl sulphate combined with fluoride on acid formation by dental plaque *in vivo.*" *Caries Research* 38: 430-435.

Gilbert, P. (1975). The antimicrobial activity of some glycolmonophenyl ethers. Pharmacy, Sunderland Polytechnic. Doctor of Philosophy.

Gilbert, P., A. J. McBain, R. G. Ledder, R. G. Bartolo, C. E. Catrenich and D. Charbonneau (2003). "Effects of triclosan-containing rinse on the dynamics and antimicrobial susceptibility of *in vitro* plaque ecosystems." *American Society for Microbiology* 47(11): 3531-3538.

Gilbert, P., A. J. McBain, R. G. Ledder and P. Sreenivasan (2004). "Selection for high-level resistance by chronic triclosan exposure is not universal." *Journal of Antimicrobial Chemotherapy* 53: 772-777.

Gilbert, P. and L. E. Moore (2005). "Cationic antiseptics: diversity of action under a common epithet." *Journal of Applied Microbiology* 99(4): 703-715.

Gilby, A. R. and A. V. Few (1959). "Lysis of protoplasts of *Micrococcus lysodeikticus* by ionic detergents." *Journal of General Microbiology* 23: 19-26.

Gomez , E. M. and J. Y. Maillard (2005). "Triclosan inhibition of fatty acid synthesis and its effect on growth of *Escherichia coli* and *Pseudomonas aeruginosa*." *Journal of Antimicrobial Chemotherapy* 55: 879-882.

Gordon, J. M., I. B. Lamster and M. C. Seiger (1985). "Efficacy of Listerine antiseptic in inhibiting the development of plaque and gingivitis." *Journal of Clinical Periodontology* 12: 697-704.

Gottschalk, G. (1986). Bacterial Metabolism. 2 ed. New York, Springer-Verlag.

Gray, T. (2005). "*Streptococcus anginosus* group: clinical significance of an important group of pathogens." *Clinical Microbiology* 27(20): 155-159.

Guggenheim, B., S. Shapiro, P. Schupbach and E. Giertsen (2001). "Validation of an *in vitro* biofilm model of supragingival plaque." *Journal of Dental Research* 80(1): 363-370.

Gusberti, F. A., P. Sampathkumar, B. E. Siegrist and N. P. Lang (1988). "Microbiological and clinical effects of chlorhexidine digluconate and hydrogen peroxide mouthrinses on developing plaque and gingivitis." *Journal of Periodontology* 15: 60-67.

Hall, M. J., R. F. Middleton and D. Westmacott (1983). "The fractional inhibitory concentration (FIC) index as a measure of synergy." *Journal of Antimicrobial Chemotherapy* 11: 427-433.

Hamilton, I. R. (1977). "Effects of fluoride on enzymatic regulation of bacterial carbohydrate metabolism." *Caries Research* 11: 262-291.

Hamilton, W. A. (1968). "The mechanism of the bacteriostatic action of tetrachlorosalicylanilide: a membrane-active antibacterial compound." *Journal of General Microbiology* 50(3): 441-458.

Hanke, M. T. (1940). " Studies on the local factors in dental caries. I. Destruction of plaques and retardation of bacterial growth in the oral cavity." *American Dental Associataion* 27: 1379-1393.

Hase, J. C., S. Edwardsson, J. Rundegren, R. Attstrom and E. Kelty (1998). "Six-month use of 0.2% delmopinol hydrochloride in comparison with 0.2% chlorhexidine digluconate and placebo. II. Effect on plaque and salivary microflora." *Journal of Clinical Periodontology* 25: 841–849.

Haubek, D., K. Poulsen, S. Poulsen, M. Kilian, N. Benzarti and O. Ennibi (2001). "Early-onset periodontitis in Morocco is associated with the highly leukotoxic clone of *Actinobacillus actinomycetemcomitans*." *Journal of Dental Research* 80(6): 1580-1583.

Healy, C. M. and M. Peterson (1999). "The effect of a sodium lauryl sulfate-free dentifrice on patients with recurrent oral ulceration." *Oral Diseases* 5: 39-43.

Heath, R. J., J. R. Rubin, D. R. Holland, E. Zhang, M. E. Snow and C. O. Rock (1999). "Mechanism of triclosan inhibition of bacterial fatty acid synthesis." *Journal of Biological Chemistry* 274: 11110–11114.

Hodgson, A. E. and P. Gilbert (1995). "A simple *in vitro* model for growth control of bacterial biofilms." *Journal of Applied Bacteriology* 79: 87-93.

Holbeche, J. D., M. K. Ruljancich and P. C. Reade (1975). "A clinical trial of the efficacy of a cetylpyridinium chloride-based mouthwash 1. Effect on plaque accumulation and gingival condition." *Australian Dental Journal* 20(6): 397-404.

Holt, S. C. and J. L. Ebersole (2005). "*Porphyromonas gingivalis, Treponema denticola*, and *Tannerella forsythia*: the 'red complex', a prototype polybacterial pathogenic consortium in periodontitis." *Periodontology 2000* 38: 72-122.

Holt, S. C., J. L. Ebersole, J. Felton, M. Brunsvold and K. S. Kornman (1988). "Implantation of *Bacteroides gingivalis* in nonhuman primates initiates progression of periodontitis." *Science* 239: 55-57.

Holt, S. C., L. Kesavalu, S. Walker and C. A. Genco (1999). "Virulence factors of *Porphyrornonas gingivalis*." *Periodontol 2000* 20: 168-238.

Hope, C. K., D. Clements and M. Wilson (2002). "Determining the spatial distribution of viable and nonviable bacteria in hydrated microcosm dental plaques by viability profiling." *Journal of Applied Microbiology* 93(3): 448-455.

Horowitz, H. S. (1971). "The Effect On Human Dental Caries Of Weekly Oral Rinsing With A Sodium Fluoride Mouthwash." *Archives of Oral Biology* 16: 609-616.

Hoyos, D. F., J. S. Murray and L. Shaw (1977). "The effect of chlorhexidine gel on plaque and gingivitis in children." *British Dental Journal* 142: 366-369.

Hughes, C. V., P. E. Kolenbrander, R. N. Andersen and L. V. H. Moore (1988). "Coaggregation properties of human oral *veilionella* spp.: relationship to colonization site and oral ecology." *Applied and Environmental Microbiology* 54(8): 1957-1963.

Hugo, W. B. and A. R. Longworth (1966). "The effect of chlorhexidine on the electrophoretic mobility, cytoplasmic constituents, dehydrogenase activity and cell walls of *Escherichia coli* and *Staphylococcus aureus*." *Journal of Pharmaceutical Pharmacology* 18: 569–578.

Humphrey, S. P. and R. T. Williamson (2001). "A review of saliva: Normal composition, flow, and function." *The Journal of Prosthetic Dentistry* 85(2): 162-169.

Imlay, J. A. (2002). How oxygen damages microbes: oxygen tolerance and obligate anaerobiosis. *Advances in Microbial Physiology* 46**:** 111-153.

Jenkins, S., M. Addy and R. Newcombe (1991). "Triclosan and sodium lauryl sulphate mouthwashes." *Journal of Clinical Periodontology* 18: 140-144.

Jenkins, S., M. Addy and R. Newcombe (1993). "The effects of a chlorhexidine toothpaste on the development of plaque, gingivitis and tooth staining." *Journal of Clinical Periodontology* 20(1): 59-62.

Jenkinson, H. F. and R. J. Lamont (1997). "Streptococcal adhesion and colonization." *Critical Reviews in Oral Biology and Medicine* 8: 175–200.

Jian, W. and X. Dong (2002). "Transfer of *Bifidobacterium inopinatum* and *Bifidobacterium denticolens* to *Scardovia inopinata* gen. nov., comb. nov., and *Parascardovia denticolens* gen. nov., comb. nov., respectively." *International Journal of Systematic and Evolutionary Microbiology* 52: 809-812.

Jolivet-Gougeon, A. and J.-L. Sixou (2007). "Antimicrobial treatment of capnocytophaga infections." *International Journal of Antimicrobial agents* 29(367-373).

Jones, R. D., H. B. Jampani and J. L. Newman (2000). "Ticlosan: A review of effectiveness and safety in health care settings." *American Journal of Infection Control* 28(2): 184-196.

Jordan, H. V. and P. H. (1972). "Periodontal lesions in hamsters and gnotobiotic rats infected with actinomyces of human origin." *Journal of Periodontal Research* 7(1): 21-28.

Kanzor, C. E., W. J. Loesche, P. M. Mitchell, A. M. Lee, L. N. Stokes, F. E. Dewhirst and B. J. Paster (2002). "Diversity of bacterial populations on the tongue dorsa of patients with halitosis and healthy patients." *American Society for Microbiology* 41(2): 558-563.

Kaplan, J. and D. H. Fine (2002). "Biofilm Dispersal of *Neisseria subflava* and other phylogenetically diverse oral bacteria." *American Society for Microbiology* 68(10): 4943-4950.

Keevil, C. W., D. J. Bradshaw, A. B. Dowsett and T. W. Feary (1987). "Microbial film formation: dental plaque deposition on acrylic tiles using continuous culture techniques." *Journal of Applied Microbiology* 62(2): 129-138.

Klein, I. M. and B. Goncalves (2003). "Detection of *Tannerella forsythensis (Bacteroides forsythus)* and *Porphyromonas gingivalis* by polymerase chain reaction in subjects with different periodontal status." *Journal of Periodontology* 74(6): 798-802.

Kolenbrander, P. E., R. N. Andersen, K. M. Kazmerak and R. J. Palmer (2000). Coaggregation and coadhesion in oral biofilms. Community Structure and Co-Operation in Biofilms, Cambridge, Lappin- Scott H. M.

Kolenbrander, P. E. and J. London (1993). "Adhere today, here tomorrow: oral bacterial adherence." *Journal of Bacteriology* 175: 3247–3252.

Kondo, W., M. Sato and N. Sato (1978). "Properties of the human salivary aggregating factor for *Leptotrichia buccalis* cells." *Archives in Oral Biology* 23: 453-458.

Kononen, E., H. Jousimies-Somer, A. Bryk, M. Kilian and T. Kilpi (2002). "Establishment of streptococci in the upper respiratory tract: longitudinal changes in the mouth and nasopharynx up to 2 years of age." *Journal of Medical Microbiology* 51: 723-730.

Kornberg, H. L. (1965). "The role and control of the glyoxylate cycle in *Escherichia coli*." *Biochimestry Journal* 99: 1-11.

Kuramitsu, H. K. and R. P. Ellen (2000). Oral bacterial ecolgy: the molecular basis. horizon scientific press.

Kurbert, D., M. Rubin, M. L. Barnett and J. W. vincent (1993). "Antiseptic mouthrinse-induced microbial cell surface alterations." *American Journal of Dentistry* 6(6): 277-279.

Lang, N. P. and H. N. Newman (1997). Chemicals in Periodontics. Proceedings of the 2nd European Workshop on Periodontology, Berlin.

LeChevallier, M., C. D. Cawthon and R. L. Lee (1988). "Inactivation of Biofilm Bacteria." *Applied and Environmental Microbiology* 54(10): 2492-2499.

Ledder, R. G., P. Gilbert, A. Pluen, P. K. Sreenivasan, W. De Vizio and A. J. McBain (2006). "Individual microflora beget unique oral microcosms." *Journal of Applied Microbiology* 100(5): 1123-1131.

Ledder, R. G., T. Madhwani, P. Sreenivasan, W. De Vizio and A. J. McBain (2009). "An *in vitro* evaluation of hydrolytic enzymes as dental plaque control agents." *Journal of Medical Microbiology* 58(4): 482-491.

Ledder, R. G., A. J. McBain, P. Gilbert, P. Sreenivasan and W. De Vizio (2008). Effects of Colgate-Total® and Crest Pro-Health® on oral microcosms. The International Association for Dental Research 86th General Session & Exhibition, Toronto, Ontario.

Lee, S. M., S. Y. Yoo, Y. Yoon, H. Kim and K. Kim (2005). "Prevalence of putative periodontopathogens in subgingival dental plaques from gingivitis lesions in korean orthodontic patients." *The Microbiological Society of Korea* 43(3): 260-265.

Lewin, R. and W. T. Hughes (1966). "*Neisseria subflava* as a cause of meningitis and septicemia in children." *Journal of the American medical association* 195(10): 821-823.

Loe, H. and C. R. Schiott (1970). "The effect of mouthrinses and topical application of chlorhexidine on the development of dental plaque and gingivitis in man." *Journal of Peridontal Research* 5: 79–83.

Löe, H., E. Theilade, S. B. Jensen and C. R. Schiøtt (1967). "Experimental gingivitis in man." *Journal of periodontal Research* 2(4): 282-289.

Loesche, W. J. (1986). "Role of *Streptococcus mutans* in human dental decay." *American society for microbiology* 50(4): 353-380.

Loesche, W. J. (1992). "The specific plaque hypothesis and the antimicrobial treatment of periodontal disease." *Dental update* 19(2): 68-74.

Loesche, W. J. and S. Syed (1978). "Bacteriology of human experimental gingivitis: effect of plaque age." *Infection and Immunity* 21(3): 821-829.

Longworth, A. R. (1971). Chlorhexidine. In Inhibition and Destruction of the Microbial Cell. London, Academic Press.

Macuch, P. J. and A. Tanner (2000). "*Campylobacter* species in health, gingivitis, and periodontitis." *Journal of Dental Research* 72(2): 785-792.

Maeda, N. (1980). "Anaerobic, gram-positive, pleomorphic rods in human gingival crevice." *The Bulletin of Tokyo Medical and Dental University* 277(1): 63-70.

Mah, T.-F. C. and G. O'Toole (2001). "Mechanisms of biofilm resistance to antimicrobial agents." *Trends in Microbiology* 9(1): 34-39.

Maiden, M. F., A. Tanner, P. J. Macuch, J. S. Murray and R. L. J. Kent (1998). "Subgingival temperature and microbiota in initial periodontitis." *Journal of Clinical Periodontology* 25: 786-793.

Maltz, M. and C. G. Emilson (1982). "Susceptibility of oral bacteria to various fluoride salts." *Journal of Dental Research* 61(6): 786-790.

Mandel, I. D. (1988). "Chemotherapeutic agents for controlling plaque and gingivitis." *Journal of Clinical Periodontology* 15(8): 488-498.

Mankodi, S., N. M. Ross and K. Mostler (1987). "Clinical efficacy of Listerine in inhibiting and reducing plaque and experimental gingivitis." *Journal of Clinical Periodontology* 14: 285-288.

Marquis, R. E., J. Sheng and T. M. Nguyen (2005). "Multi-target antimicrobial actions of zinc against oral anaerobes." *Archives in Oral Biology* 50: 747-757.

Marsh, P. (2004). "Dental plaque as a microbial biofilm." *Caries Research* 38: 204–211.

Marsh, P. D. (1989). "Host defenses and microbial homeostasis: role of microbial interactions." *Journal of Dental Research* 68: 1567-1575.

Marsh, P. D. (1991). "Sugar, fluoride, pH and microbial homeostasis in dental plaque." *Proceedings of the Finnish Dental Society* 87: 515-525.

Marsh, P. D. (1992). "Microbiological aspects of the chemical control of plaque and gingivitis." *Journal of Dental Research* 71(7): 1431-1438.

Marsh, P. D. (1994). "Microbial ecology of dental plaque and its significance in health and disease." *Advances in Dental Research* 8(2): 263-271.

Marsh, P. D. (1995). "The role of continuous culture in modelling the human microflora." *Journal of Chemical Technology & Biotechnology* 64(1): 1-9.

Marsh, P. D. (2000). "Role of the oral microflora in health " *Microbial Ecology In Health and Disease* 12: 130–137.

Marsh, P. D. (2003). "Are dental diseases example of ecological catastrophes?" *Microbiology* 149: 279-294.

Marsh, P. D. (2005). "Dental plaque: biological significance of a biofilm and community life-style." *Journal of Periodontology* 32 (suppl. 6): 7-15.

Marsh, P. D. and D. J. Bradshaw (1999). Microbial community aspects of dental plaque oral biofilms in health and disease, Cardiff.

Marsh, P. D., D. J. Bradshaw and A. S. McKee (1988). "Effects of Carbohydrate Pulses and pH on Population Shifts within Oral Microbial Communities in vitro." *Journal of Dental Research* 68(9): 1298-1302.

Marsh, P. D., J. R. Hunter, G. H. Bowden, I. R. Hamilton, A. S. McKEE, J. M. Hardie and D. C. Ellwood (1983). "The Influence of growth rate and nutrient limitation on the microbial composition and biochemical properties of a mixed culture of oral bacteria grown in a chemostat." *Journal of General Microbiology* 129(3): 755-770.

Marsh, P. D. and M. V. Martin (1999). Oral Microbiology, 4 ed. Wright.

McBain, A. J. (2009). Chapter 4: *In Vitro* Biofilm Models: an overview. advances in applied microbiology. I. L. Allen, S. Sima and M. G. Geoffrey, Academic Press. 69**:** 99-132.

McBain, A. J., R. G. Bartolo, C. E. Catrenich, D. Charbonneau, R. G. Ledder and P. Gilbert (2003). "Effects of a chlorhexidine gluconate-containing mouthwash on the vitality and antimicrobial susceptibility of in vitro oral bacterial ecosystems." *Applied and Environmental Microbiology* 69(8): 4770-4776.

McBain, A. J., R. G. Bartolo, C. E. Catrenich, D. Charbonneau, R. G. Ledder and P. Gilbert (2003). "Effects of triclosan-containing rinse on the dynamics and antimicrobial susceptibility of in vitro plaque ecosystems." *Antimicrobial Agents and Chemotherapy* 47(11): 3531-3538.

McBain, A. J., R. G. Bartolo, C. E. Catrenich, D. Charbonneau, R. G. Ledder and P. Gilbert (2003). "Growth and molecular characterization of dental plaque microcosms." *Journal of Applied Microbiology* 94(4): 655-664.

McBain, A. J., P. Gilbert and R. G. Ledder (2002). "Growth and molecular charecterization of dental plaque microcosms." *Journal of Applied Microbiology* 94: 655-664.

McBain, A. J., P. Gilbert, R. G. Ledder, C. H. Sissons, P. Sreenivasan and W. De Vizio (2005). "Development and characterization of a simple perfused oral microcosm." *Journal of Applied Microbiology* 98: 624-634.

McBain, A. J., R. G. Ledder, P. Sreenivasan and W. De Vizio (2010). "Evaluation of the specificity and effectiveness of selected oral hygiene actives in salivary biofilm microcosms." *Journal of medical microbiology* Paper in press.

McBain, A. J., R. G. Ledder and A. S. Timperley (2008). "Coaggregation between and among human intestinal and oral bacteria." *Federation of European Microbiological Societies and Microbiol Ecology* 66: 630-636.

McDermid, A. S., A. S. McKee, D. C. Ellwood and P. D. Marsh (1986). "The effect of lowering the pH on the composition and metabolism of a community of nine oral bacteria grown in a chemostat." *Journal of General Microbiology* 132(5): 1205-1214.

McFarland, L. V. (2000). "Normal flora: Diversity and functions." *Microbial Ecology in Health and Disease* 12: 193–207.

McMurry, L. M., M. Oethinger and S. B. Levy (1998). "Triclosan targets lipid synthesis." *Nature* 394: 531-532.

McNamara, T. F., J. F. Alexander and M. Lee (1972). "The role of microorganisms in the production of oral malodor." *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontology Journal* 34(1): 41-48.

Meskin, L. H. (1996). "A breath of fresh air." *Journal of American Dental Association* 127: 1282-1286.

Milnes, A. R. and G. H. Bowden (1985). "The microflora associated with developing lesions of nursing caries." *Caries Research* 19: 289-297.

Minah, G. E., E. S. Solomon and K. Chu (1985). "The association between dietary sucrose consumption and microbial population shifts at six oral sites in man." *Archives in Oral Biology* 30(5): 397-401.

Miyasaki, K. T., R. J. Genco and M. Wilson (1986). "Antimicrobial properties of hydrogen peroxide and sodium bicarbonate individually and in combination against selected oral, gram-negative, facultative bacteria." *Journal of Dental Research* 65(9): 1142-1148.

Moore, W. E. C., L. V. Holdeman, P. E. Cato, I. J. Good, E. P. Smith, R. R. Ranney and K. G. Palcanis (1984). "Variation in periodontal floras." *American Society for Microbiology* 46(3): 720-726.

Moore, W. E. C. and L. V. H. Moore (1994). "The bacteria of periodontal diseases." *Periodontology 2000* 5: 66–77.

Morris, A. J. and J. Steele (1998). "The oral cleanliness and periodontal health of UK adults in 1998." *British Dental Journal* 191: 186-192.

Moskowitz, S. M., J. M. Foster, J. Emerson and J. L. Burns (2004). "Clinically feasible biofilm susceptibility assay for isolates of *Pseudomonas aeruginosa* from patients with cystic fibrosis." *Journal of Clinical Microbiology* 42(5): 1915-1922.

Newman, H. N. (1990). "Plaque and chronic inflammatory periodontal disease a question of ecology." *Journal of Clinical Periodontology* 17(8): 533-541.

Nolte, W. A. (1982). Oral microbiology with basic microbiology and immunology, 4 ed. The C. V. Mosby company.

Nørskov-Lauritsen, N. and M. Kilian (2006). "Reclassification of *Actinobacillus actinomycetemcomitans, Haemophilus aphrophilus, Haemophilus paraphrophilus* and *Haemophilus segnis* as *Aggregatibacter actinomycetemcomitans* gen. nov., comb. nov., *Aggregatibacter aphrophilus* comb. nov. and *Aggregatibacter segnis* comb. nov., and emended description of *Aggregatibacter aphrophilus* to include V factor-dependent and V factor independent isolates." *International Journal of Systematic and Evolutionary Microbiology* 56: 2135-2146.

Novick, A. and L. Szilard (1951). "Experiments on spontaneous and chemically induced mutations of bacteria growing in the chemostat." *Cold Spring Harbor Symposia on Quantitative Biology* 16: 337-343.

Ogaard, B., L. Seppa and G. Rolla (1994). "Professional topical fluoride applications: clinical efficacy and mechanism of action." *Advances in Dental Research* 8(2): 190-201.

Oppermann, R. V., G. Rölla, J. R. Johanses and S. Assev (1980). "Thiol groups and reduced acidogenicity of dental plaque in the presence of metal ions *in vivo.*" *Scandinavian Journal of Dental Research* 88: 389-396.

Ouhayoun, J. P. (2003). "Penetrating the plaque biofilm: impact of essential oil mouthwash." *Journal of Clinical Periodontology* 30 (Suppl. 5): 10-12.

Pan, P., M. L. Barnett, J. Coelho, C. Brogdon and M. B. Finnegan (2000). "Determination of the *in situ* bactericidal activity of an essential oil mouthrinse using a vital stain method." *Journal of Clinical Periodontology* 27(4): 256-261.

Parveen, A., G. Smith, V. Salisbury and S. M. Nelson (2001). "Biofilm culture of *Pseudomonas aeruginosa* expressing lux genes as a model to study susceptibility to antimicrobials." *Federation of European Microbiological Societies Microbiology Letters* 199(1): 115-118.

Paster, B. J., S. K. Boches, J. L. Galvin, R. E. Ericson, C. N. Lau, V. A. Levanos, A. Sahasrabudhe and F. E. Dewhirst (2001). "Bacterial diversity in human subgingival plaque." *Journal of Bacteriology* 183: 3770–3783.

Perea, S., G. Gonzalez, A. W. Fothergill, W. R. Kirkpatrick, M. G. Rinaldi and T. F. Patterson (2002). "*In vitro* interaction of caspofungin acetate with voriconazole against clinical isolates of Aspergillus spp." *Antimicrobial Agents and Chemotherapy* 46(9): 3039-3041.

Peters, A. C. and J. W. T. Wimpenny (1988). "A constant-depth laboratory model film fermentor." *Biotechnology and Bioengineering* 32(3): 263-270.

Pfaller, M. A., C. Grant, V. Morthland and J. Rhine-Chalberg (1994). "Comparative evaluation of alternative methods for broth dilution susceptibility testing of fluconazole against *Candida albicans.*" *Journal of Clinnical Microbiology* 32(2): 506-509.

Pianotti, R., S. Lachette and S. Dills (1986). "Desulfuration of cysteine and methionine by *Fusobacterium nucleatum.*" *Journal of Dental Research* 65(913-917).

Pollack, S. and A. Mogtader (1984). "*Neisseria subflava* endocarditis; case report and review of the literature." *The American Journal of Medicine* 76: 752-758.

Pratten, J., A. W. Smith and M. Wilson (1998). "Response of single species biofilms and microcosm dental plaques to pulsing with chlorhexidine." *Journal of Antimicrobial Chemotherapy* 42(4): 453-459.

Pratten, J., K. Wills, P. Barnett and M. Wilson (1998). "*In vitro* studies of the effect of antiseptic-containing mouthwashes on the formation and viability of *Streptococcus sanguis* biofilms." *Journal of Applied Microbiology* 84(6): 1149-1155.

Pratten, J. and M. Wilson (1999). "Antimicrobial susceptibility and composition of microcosm dental plaques supplemented with sucrose." *Antimicrobial Agents and Chemotherapy* 43(7): 1595-1599.

Rasiah, I. A., L. Wong, S. A. Anderson and C. H. Sissons (2005). "Variation in bacterial DGGE patterns from human saliva: over time, between individuals and in corresponding dental plaque microcosms." *Archives of Oral Biology* 50(9): 779-787.

Rawlinson, A., S. Pollington, T. F. Walsh and D. Lamb (2008). "Efficacy of two alcohol-free cetylpyridinium chloride mouthwashes – a randomized double-blind crossover study." *Journal of Clinical Periodontology* 35: 230-235.

Reig, M., F. Baquero, M. Gracia and E. Loza (1985). "*Leptotrichia buccalis* bacteremia in neutropenic children." *American Society for Microbiology* 22(2): 320-321.

Ritchie, J. A. and C. L. Jones (1988). The inhibition of facultative and obligately anaerobic bacteria by triclosan. "Anaerobes today" J. M. Hardie, Borriello S. P. editors. Chichester: Wiley, pp. 240-241.

Robinson, C., J. Kirkham, R. Percival, R. C. Shore, W. A. Bonass, S. J. Brookes, L. Kusa, H. Nakagaki, K. Kato and B. Nattress (1997). "A method for the quantitative site-specific study of the biochemistry within dental plaque biofilms formed *in vivo*." *Caries Research* 31: 194–200.

Robrish, S. A., C. Oliver and J. Thompson (1991). "Sugar metabolism by fusobacteria: regulation of transport, phosphorylation, and polymer formation by *Fusobacterium mortiferum* ATCC 25557." *Infection and Immunity* 59: 4547-4554.

Robrish, S. A. and J. Thompson (1988). "Suppression of polyglucose degradation in *Fusobacterium nucleatum* ATCC 10953 by amino acids." *Federation of European Microbiological Societies Microbiology Letters* 55: 29-34.

Rocas, I. N. and J. F. Siqueira (2005). "Occurrence of two newly named oral treponemes - *Treponema parvum* and *Treponema putidum* - in primary endodontic infections." *Oral Microbiology and Immunology* 20(6): 372-375.

Rogers, A. H., Zilm, P. S., N. J. Gully, A. L. Pfennig and P. D. Marsh (1991). "Some aspects of the growth and metabolism of *Fusobacterium nucleatum* ATCC 10953 in continuous culture." *Oral Microbiology and Immunology.* 6: 250-255.

Roldan, S., D. Herrera and M. Sanz (2003). "Biofilms and the tongue: therapeutical approaches for the control of halitosis." *Clinical Oral Investigations* 7: 189–197.

Rolla, G., B. Ogaard, K. Skjörland and S. M. Waler (1993). "Effects of oral rinsing with tridosan and sodium lauryl sulfate on dental plaque formation: a pilot study." *Journal of Dental Research* 101: 192-195.

Rosenberg, M. (2002). "The science of bad breath." *Science of America* 4: 72-79.

Roslev, R. and G. M. King (1993). "Application of a tetrazolium salt with a water-soluble formazan as an indicator of viability in respiring bacteria." *Applied Environmental Microbiology* 59(9): 2891-2896.

Rosling, B., G. Dahlé, A. Volpe, Y. Furuichi, P. Ramberg, P. Lindhe (1997). "Effect of triclosan on the subgingival microbiota of periodontitis-susceptible subjects." *Journal of Clinical Periodontology* 24(12): 881-887.

Russell, J. B. (1992). "Another explanation for the toxicity of fermentation acids at low pH: anion accumulation versus uncoupling." *Journal of Applied Microbiology* 73(5): 363-370.

Sanders, W. E. and C. C. Sanders (1984). Modification of normal flora by antibiotics: Effects on individuals and the environment. New York, Churchill Livingstone.

Sanyal, B. and C. Russell (1978). "Nonsporing, anaerobic, Gram-positive rods in saliva and the gingival crevice of humans." *American Society for Microbiology* 35(4): 670-678.

Sanz, M., S. Roldan and D. Herrera (2001). "Fundamentals of breath malodour." *Journal of Contemporary Dental Research* 2(4): 1-17.

Scardovi, V. and F. Crociani (1974). "*Bifidobacterium catenulatum, Bifidobacterium dentium,* and *Bifidobacterium angulatum*: three new species and their deoxy ribonucleic acid homology relationships" *International Journal of Systematic Bacteriology* 42(1): 6-20.

Scott, M. G., H. Yan. and R. E. Hancock (1999). "Biological Properties of Structurally Related a-Helical Cationic Antimicrobial Peptides." *Infection and Immunity* 67(4): 2005-2009.

Seymour, G. J., P. J. Ford, M. P. Cullinan, S. Leishman and K. Yamazaki (2007). "Relationship between periodontal infections and systemic disease." *Clinical Microbiology and Infection* 13: 3-10.

Shafa, F. and M. R. J. Salton (1960). "Disaggregation of bacterial cell walls by anionic detergents." *Journal of General Microbiology* 22: 137-141.

Shah, H. N. and S. E. Gharbia (1989). "Ecological events in subgingival dental plaque with reference to *Bacteroides* and *Fusobacterium* species." *Infection* 17: 264-268.

Shah, H. N. and S. E. Gharbia (1993). "Studies on the physiology and ecology of black-pigmented gram-negative anaerobes which may be important in disease development." *Federation of European Microbiological Societies Microbiology Letters* 6: 165-172.

Shah, H. N. and R. A. D. Williams (1976). "Comparison of the biochemical properties of Bacteroides melinogenicus from human dental plaque and other sites." *Journal of Applied Bacteriology* 41: 473-492.

shah, H. N. and R. A. D. Williams (1987). "Utilization of glucose and amino acids by *Bacteroides intermedius* and *Bacteroides gingivalis.*" *Current Microbiology* 15: 241-246.

Shah, N. B. (1982). Zinc derivatives and their use in dental compositions. U. S. Patent. United States of America, Richardson-Vicks Inc. 4325939.

Shapira, L., P. Tepper and D. Steinberg (2000). "The interactions of human neutrophils with the constituents of an experimental dental biofilm." *Journal of Dental Research* 79: 1802–1807.

Shapiro, S., E. Giertsen and B. Guggenheim (2002). "An *in vitro* oral biofilm model for comparing the efficacy of antimicrobial mouthrinses." *Caries Research* 36(2): 93-100.

Sharma, N. C., C. H. Charles, H. J. Galustians, J. Qaqish, J. A. McGuire and J. W. Vincent (2001). "Comparative efficacy of an antiseptic mouthrinse and an antiplaque/ antigingivitis dentifrice." *Journal of American Dental Association* 132(670-675).

Sharma, N. C., C. H. Charles, M. C. Lynch, J. Qaqish, J. A. McGuire, H. J. Galustians and L. D. Kumar (2004). "Adjunctive benefit of an essential oil–containing mouthrinse in reducing plaque and gingivitis in patients who brush and floss regularly." *Journal of American Dental Association* 135(496-504).

Shu, M., L. Wong, J. H. Miller and C. H. Sissons (1999). "Development of multi-species consortia biofilms of oral bacteria as an enamel and root caries model system." *Archives of Oral Biology* 45: 27-40.

Skjörland, K., P. Gjermo and G. Rölla (1978). "Effect of some polyvalent cations on plaque formation in *vivo.*" *Scandinavian Journal of Dental Research* 86: 103-107.

Slater, T. F. and B. Sawyer (1963). "Sudies on succinate-tetrazolium reductase systems iii. points of coupling of four different tetrazolium salts." *Biochim. Biophys. Acta* 77: 383-393.

Slots, J. (1977). "The predominant cultivable microflora of advanced periodontitis." *Journal of Dental Research* 85: 114-121.

Slots, J. and M. A. Listgarten (1988). "*Bacteroides gingivalis*, *Bacteroides intermedius* and *Actinobacillus actinomycetemcomitans* in human periodontal diseases." *Journal of Clinical Periodontology* 15: 85-93.

Slots, J. and M. Ting (1999). "*Actinobacillus actinomycetemcomitans* and *Porphromonas gingivalis* in human periodontal disease: occurrence and treatment." *Periodontology 2000* 20: 82-121.

Smith, A. J., M. Jackson and J. Bagg (2001). "The ecology of Staphylococcus species in the oral cavity." *Journal of medical microbiology* 50: 940-946.

Smith, J. J. and G. A. McFeters (1997). "Mechanisms of INT (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride), and CTC (5-cyano-2,3-ditolyl tetrazolium chloride) reduction in *Escherichia coli* K-12." *Journal of Microbiological Methods* 29: 161–175.

Socransky, S. (1977). "Microbiology of periodontal disease-present status and future considerations." *Journal of Periodontology* 48: 497-504.

Socransky, S. and A. Haffajee (2005). "Periodontal microbial ecology." *Periodontology 2000* 38: 135-187.

Socransky, S. and S. D. Manganiello (1971). "The oral microbiota of man from birth to senility." *Journal of Periodontology* 42(8): 485-494.

Socransky, S. and S. D. Manganiello (1971). "The oral microbiota of man from birth to senility." *Journal of Periodontology* 42(8): 485-496.

Spratt, D. A., A. J. Weightman and W. G. Wade (1999). "Diversity of oral asaccharolytic Eubacterium species in periodontitis-identification of novel phylotypes representing uncultivated taxa." *Oral Microbiology and Immunology* 14(1): 56-59.

Sreenivasan, P. and A. Gaffar (2002). "Antiplaque biocides and bacterial resistance: a review." *Journal of Clinical Periodontology* 29: 965–974

Stephen, K. W., C. A. Saxton, C. L. Jones, J. A. Ritchie and T. Morrison (1990). "Control of gingivitis and calculus by a dentifrice containing a zinc salt and triclosan." *Journal of Periodontology* 61: 674–679.

Suarez, F. L., J. K. Furne, J. Springfield and M. D. Levitt (2000). "Morning breath odor: influence of treatments on sulfur gases." *Journal of Dental Research* 79(10): 1773-1777.

Sudjalim, T. R., M. G. Woods, D. J. Manton and E. C. reynolds (2007). "Prevention of demineralization around orthodontic brackets *in vitro*." *Journal of Orthodontics and Dentofacial Orthopedics* 131(6): 705.e1-705.e9.

Sukontapatipark, W., M. A. El-Agroudi, N. J. Selliseth, K. Thunold and K. A. Selvig (2001). "Bacterial colonization associated with fixed orthodontic appliances. A scanning electron microscopy study." *The European Journal of Orthodontics* 23(5): 475-484.

Suller, M. T. E. and A. D. Russell (2000). "Triclosan and antibiotic resistance in *Staphylococcus aureus*." *Journal of Antimicrobial Chemotherapy* 46(1): 11-18.

Sunde, P. T., I. Olsen, P. O. Lind and L. Tronstad (2000). "Extraradicular infection: a methodological study." *Journal of endodontics & dental traumatology* 16: 84-90.

Sundqvist, G. (1992). "Associations between microbiai species in dental root canal infections." *Oral Microbiology and Immunology* 7: 257-262.

Svatun, B., C. A. Saxton and G. Rolla (1990). "Six-month study of the effect of a dentifrice containing zinc citrate and triclosan on plaque, gingival health, and calculus." *Scandinavian Journal of Dental Research* 98: 301-304.

Svatun, B., C. A. Saxton, F. van der Ouderaa and G. Rölla (1987). "The influence of a dentifrice containing a zinc salt and a nonionic antimicrobial agent on the maintenance of gingival helath." *Journal of Clinical Periodontology* 14(8): 457-461.

Takazoe, I., T. Matsukubo and T. Katow (1978). "experimental formation of "corn cob" *in vitro*." *Journal of Dental Research* 57(2): 384-387.

Tanner, A., R. Kent, M. F. J. Maiden and M. A. Taubman (1996). "Clinical, microbiological and immunological profile of healthy, gingivitis and putative active periodontal subjects." *Journal of periodontal Research* 31(3): 195-204.

Tanner, A., M. F. Maiden, P. J. Macuch, L. L. Murray and R. L. Kent (1998). "Microbiota of health, gingivitis, and initial periodontitis." *Journal of Clinical Periodontology* 25(2): 85-98.

Taylor, P. C., F. D. Schoenknecht, J. C. Sherris and E. C. Linner (1983). "Determination of minimum bactericidal concentrations of oxacillin for *Staphylococcus aureus*: influence and significance of technical factors." *Antimicrobial Agents and Chemotherapy* 23(1): 142-150.

Theilade, E. (1986). "The non-specific theory in microbial etiology of inflammatory periodontal diseases." *Journal of Clinical Periodontology* 13(10): 905-911.

Tinanoff, N. and D. A. Camosci (1984). "Anti-bacterial determinants of stannous fluoride." *Journal of Dental Research* 63: 1121-1125.

Tinanoff, N. and R. L. Zameck (1987). "Effects of naf and snf$_2$ on growth, acid and glucan production of several oral streptococci." *Archives in Oral Biology* 32(11): 807-810.

Tonzetich, J. (1977). "Production and origin of oral malodor: a review of mechanisms and methods of analysis." *Journal of Periodontology* 48: 13-20.

van Houte, J. (1980). "Bacterial specificity in the etiology of dental caries." *International Dental Journal* 300(4): 305-326.

van Houte, J. (1994). "Role of microorganisms in caries etiology." *Journal of Dental Research* 73(3): 672-681.

van Houte, J., R. J. Gibbons and A. Pulkkinen (1972). "Ecology of human oral Lactobacilli." *Infection and Immunity* 6(5): 723-729.

Van Palenstien, W. H. and M. Helderman (1983). "A selective medium for the two major subgroups of the bacterium *Streptococcus mutans* isolated from human dental plaque and saliva." *Archives in Oral Biology* 28: 599-603.

Verran, J., P. Airey, A. Packer and K. A. Whitehead (2008). Chapter 8: Microbial retention on open food contact surfaces and implications for food contamination. *Advances in Applied Microbiology*. S. S. Allen I. Laskin and M. G. Geoffrey, Academic Press. 64**:** 223-246.

Waler, S. M. (1997). "The effect of some metal ions on volatile sulfur-containing compounds originating from the oral cavity." *Acta Odontol Scand* 55: 261-264.

Weber, D. A., K. Howard-Nordan, B. A. Buckner, S. A. Helsinger, R. A. Lueders, L. K. Court, B. W. Bollmer, M. A. Perlich and L. K. Sewak (1995). "Microbiological assessment of an improved stannous fluoride dentifrice." *Journal of Clinical Dentistry* 6: 97–104.

Wennstorm, J. and J. Lindhe (1979). "Effect of hydrogen peroxide on developing plaque and gingivitis in man." *Journal of Clinical Periodontology* 6: 115-130.

White, D. J. and G. H. Nancollas (1990). "Physical and chemical considerations of the role of firmly and loosely bound fluoride in caries prevention." *Journal Dental Research* 69: 587-594.

Whittaker, C. J., C. M. Klier and P. E. Kolenbrander (1996). "Mechanisms of adhesion by oral bacteria " *Annual Reviews in Microbiology* 50: 513–552.

Wilson, M. (2005). Microbial inhabitants of humans: their ecology and role in health and disea, Cambridge University.

Wilson, M. and B. Henderson (1998). "Commensal communism and the oral cavity." *Journal of Dental Research* 77(9): 1674-1683.

Winkel, E. G., S. Roldan, D. Herrera, M. Sanz and A. J. Winkelhoff (2003). "Clinical effects of a new mouthrinse containing chlorhexidine, cetylpyridinium chloride and zinc-lactate on oral halitosis." *Journal of Clinical Periodontology.* 30: 300-306.

Wirthlin, M. R., P. K. Chen and C. I. Hoover (2005). "A laboratory model biofilm fermenter: design and initial trial on a single species biofilm." *Journal of Periodontology* 76(9): 1443-1449.

Woldringh, C. L. and W. van Iterson (1972). "Effects of treatment with sodium dodecyl sulfate on the ultrastructure of *E. coli.*" *American Society for Microbiology* 11(3): 801-813.

Wood, S., D. Metcalf, D. Devine and C. Robinson (2006). "Erythrosine is a potential photosensitizer for the photodynamic therapy of oral plaque biofilms." *Journal of Antimicrobial Chemotherapy* 57(4): 680-684.

Wood, S. R., J. Kirkham, P. D. Marsh, R. C. Shore, B. Nattress and C. Robinson (2000). "Architecture of intact natural human plaque biofilms studied by confocal laser scanning microscopy." *Journal of Dental Research* 79: 21–27.

Xie, H., S. Cai and R. J. Lamont (1997). "Environmental regulation of fimbrial gene expression in *Porphyromonas gingivalis.*" *American Society for Microbiology* 65(6): 2265–2271.

Xu, K. D., P. S. Stewart, F. Xia, C.-T. Huang and G. A. McFeters (1998). "Spatial physiological heterogeneity in *Pseudomonas aeruginosa* biofilm is determined by oxygen availability." *Applied Environmental Microbiology* 64(10): 4035-4039.

Yaegaki, K. and T. Suetaka (1989). "The effect of zinc chloride mouthwash on the production of oral malodour, the degradations of salivary cellular elements and proteins." *Journal of dental health* 39: 377-386.

Yang, H. W. (2004). "Occurrence of *Porphyromonas gingivalis* and *Tannerella forsythensis* in periodontally diseased and healthy subjects." *Journal of Periodontology* 75(8): 1077-1083.

Young, A., G. Jonski, G. Rolla and S. M. Waler (2001). "Effects of metal salts on the oral production of volatile sulfur-containing compounds (VSC)." *Journal of Clinical Periodontology* 28: 776–781.

Zambon, J. J., H. S. Reynolds, R. G. Dunford, W. DeVizio, A. R. Volpe, R. Berta, J. P. Tempro and Y. Bonta (1995). "Microbial alterations in supragingival dental plaque in response to a triclosan-containing dentifrice." *Oral Microbiology and Immunology* 10: 247–255.

Zimmermann, R., R. Iturriaga and J. Becker-Birck (1978). "Simultaneous determination of the total number of aquatic bacteria and the number thereof involved in respiration." *Applied and Environmental Microbiology* 36: 926-935.