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Effects of children's toothpastes and mouth rinses on *Streptococcus mutans, Streptococcus sanguinis* and *Lactobacillus acidophilus*

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Abstract

Objectives: As suppression of *mutans streptococci* in young children may prevent or delay colonisation of the oral cavity, tooth brushing with dentifrices containing anti-*Streptococcus mutans* activity may aid in preventing caries. The aims of this study were to compare the effects of children's toothpastes, mouth rinses, oral antiseptics and fluoride solutions on the growth of *Streptococcus mutans* and non-mutans bacteria (*Streptococcus sanguinis and Lactobacillus acidophilus*).

Methods: The agar diffusion assay at neutral pH was used to determine bacterial growth inhibition. Zones of bacterial inhibition were measured using a micrometer gauge.

Results: Dentifrices containing 1,450 ppm fluoride produced greater growth inhibition of both *S. mutans* and *S. sanguinis* than those with < 500 ppm. No inhibition was seen for pure solutions of sodium fluoride or sodium monofluorophosphate at fluoride concentrations up to 10%. Stannous fluoride exerted anti-bacterial effects at concentrations above 1%. Significant growth inhibition of both *S. mutans* and *S. sanguinis* was seen with sodium lauryl sulphate at 0.25% and with triclosan at 0.01%.

The mouth rinses containing 2% chlorhexidine gluconate, 0.05% cetylpyridinium chloride and 0.05% sodium fluoride produced anti-bacterial effects against *S. mutans, S. sanguinis* and *L. acidophilus.* Of the pure compounds, 0.01% chlorhexidine produced the greatest zone of growth inhibition against *S. mutans*.

Of the combinations tested, 0.1% sodium fluoride with 5% povidone iodine produced synergistic anti-bacterial effects against *S. mutans* and *S. sanguinis.* The combination of 10% povidone iodine with 0.5% sodium hypochlorite produced additive anti-bacterial effects against *L. acidophilus*. Interference was seen in some combinations such as chlorhexidine with sodium lauryl sulphate, most likely through anion-cation reactions. However, 0.1% sodium fluoride when combined with 0.01% chlorhexidine did not interfere with the anti-bacterial effects of chlorhexidine alone

against *S. mutans* or *S. sanguinis*, but it reduced the anti-bacterial effects of cetyl pyridinium chloride alone against these bacteria.

Conclusion: Sodium lauryl sulphate and triclosan are major bacterial inhibitory compounds in children's dentifrices. Mouth rinses containing chlorhexidine, sodium fluoride and cetyl pyridinium chloride have growth inhibitory effects against *Streptococcus mutans*, *Streptococcus sanguinis* and *Lactobacillus acidophilus*. The combinations of povidone iodine with sodium hypochlorite and povidone iodine with sodium fluoride produced additive and synergistic effects respectively.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Publications during candidature

(1) Inhibitory effects of children's toothpastes and fluoride compounds on *Streptococcus mutans, Streptococcus sanguinis* and *Lactobacillus acidophilus* - accepted for publication in 2014

(2) Effect of antiseptic mouth rinses on S*treptococcus mutans, Streptococcus sanguinis* and *Lactobacillus acidophilus* - accepted for publication in 2014

(3) Anti-bacterial effects of combinations of oral antiseptics and sodium fluoride on S*treptococcus mutans, Streptococcus sanguinis* and *Lactobacillus acidophilus* - submitted for publication in 2014

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(1) Inhibitory effects of children's toothpastes and fluoride compounds on *Streptococcus mutans, Streptococcus sanguinis* and *Lactobacillus acidophilus* - incorporated as Chapter 3

Contributor	**Statement of contribution**
Dr Alana Evans (Candidate)	Designed experiments (75%) Wrote and edited the paper (75%)
Dr Lei Chai	Designed experiments (5%)
Prof. W. Kim Seow	Designed experiments (20%) Wrote and edited paper (20%)
Prof Laurence Walsh	Wrote and edited paper (5%)

(2) Effect of antiseptic mouth rinses on S*treptococcus mutans, Streptococcus sanguinis* and *Lactobacillus acidophilus* - incorporated as Chapter 4

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Prof Laurence Walsh	Wrote and edited paper (5%)

Contributions by others to the thesis

Professor Laurence Walsh: contributed in providing laboratory guidance to the research and in writing the publications.

Dr Shaneen Leishman: contributed in providing laboratory guidance to the research, analysis of results and in writing the publications

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List of Abbreviations

ECC - early childhood caries

MS - Mutans streptococci

S. mutans - Streptococcus mutans

S. sanguinis - Streptococcus sanguinis

L. acidophilus - Lactobacillus acidophilus

F - fluoride

NaF - sodium fluoride

MFP - monofluorophosphate

SnF_2 - stannous fluoride

CHX - chlorhexidine

CPC - cetyl pyridinium chloride

SLS - sodium lauryl sulphate

NaOCl - sodium hypochlorite

PI - povidone iodine

ppm - parts per million

Chapter One:

Background and Aims

1. Background to thesis

Worldwide, Early Childhood Caries (ECC) remains a difficult condition to control, with few practical methods for prevention. As *Streptococcus mutans* is one of the main bacteria implicated in ECC, reduction of this bacteria using anti-bacterial agents is a possible method of caries control. Tooth brushing is considered a common mode of caries prevention and is recommended to all children and adults alike in many countries, including Australia. A recent study by Pukallus[1] has shown that brushing alone can reduce the risk of caries. This is likely due to the physical disturbance of plaque which relies on good motor control of the tooth brush to engage all areas of plaque accumulation. Children are still developing these motor skills so it is important for parents to be taught how to brush their children's teeth. As children can be incompliant with tooth brushing, the use of a dentifrice containing antiseptic or remineralising agents is recommended for added benefit over brushing alone. These agents are able to produce anti-bacterial effects throughout the oral cavity due to, in the case of a dentifrice, the foaming action and do not rely solely on the physical disturbance of the plaque. This study aims to compare the anti-bacterial effects of fluoride and antiseptic compounds which have potential for clinical use against early childhood caries.

ECC is a chronic childhood disease resulting from a complex interaction between bacteria, the hosts tooth surface and carbohydrates that results in acid formation and enamel demineralisation.[2-5] ECC is defined by the American Academy of Paediatric Dentistry as the presence of one or more decayed, missing or filled tooth surfaces in any primary tooth in a child aged 71 months or younger.[2] It is of great concern due to the heavy burden it places on the child, family and the oral health system in place. If left untreated, ECC can lead to severe dental pain which is a leading symptom of paediatric hospital admissions attributable to a lack of primary dental care and financial limitations.[6] In some children, ECC can result in systemic infections and facial cellulitis, a common presentation in paediatric hospital admissions that requires extended hospital stays and extensive dental treatment under a general anaesthetic, also accruing large costs.[6]

Numerous papers report on a reduced quality of life for the child.[6-8] If left untreated, ECC can result in severe toothaches and breakdown of tooth structure. This directly affects the child's ability to eat, speak and sleep and can result in failure to thrive and poor development of language skills. Teasing can occur in a social situation causing the child embarrassment.[7] Following comprehensive dental rehabilitation, an increase in social interaction, smiling and attentiveness in school is generally seen along with improved eating, sleeping and overall growth.[9-11] Also of note, it has been shown that childhood caries experience negatively impacts caries prevalence at age 20.[12] Hence, prevention and treatment of ECC is vital to ensure long term oral health for the child.

Dentifrices are tooth cleaning agents which are commercially available in paste, powder, gel or liquid form. Essential components of common toothpastes are abrasives, binders, surfactants and humectants. In addition, fluoride compounds such as sodium fluoride (NaF), sodium monofluorophosphate (MFP) and stannous fluoride (SnF_2) have been added to toothpaste for several decades for anti-caries activity.[13] The main anti-caries action of fluoride in toothpaste is thought to be prevention of demineralisation and enhancement of remineralisation of early carious lesions.[14] However, although fluoride is well known to be bactericidal,[14] it is unclear whether any anti-*Streptococcus mutans* activity is associated with the fluoride found in toothpastes.

In Australia, fluoridated toothpaste of concentration 500 ppm is used by children.[15] At this low concentration, the risk of fluorosis is reduced while still exerting anti-caries effects.[16] However, it has been shown that standard toothpaste formulations (1000 ppm F) have greater caries preventive effects as measured by the change in decayed, missing, filled tooth surfaces from baseline.[16] For this reason, in America and Europe, this higher concentration fluoride toothpaste is used by children and adults alike, with children requiring close supervision.[17] Numerous studies have been performed examining the effects of various concentrations of fluoride on mutans

streptococci.[13, 16, 18-20] However, most of these studies are clinical and compare commercially available toothpastes.

In addition to topical fluoride, high risk children are likely to benefit from the additional use of anti-bacterial compounds.[21] Mouth rinses are another valuable topical caries preventive agent, most commonly prescribed to a high caries risk individual or to medically or physically compromised patients whose tooth brushing and flossing regime is not adequate to prevent caries. Many studies have shown a positive reduction in caries following use of an antiseptic mouth rinse in adults.[22-25] Timely removal of *S. mutans* from the mouths of young children can reduce caries risk by preventing their colonisation in the mouth, as demonstrated in studies using chlorhexidine (CHX) varnish,[26] CHX gel,[27] and povidone iodine (PI) solution.[28] Common oral antiseptics are CHX, cetyl pyridinium chloride (CPC), PI and essential oils (EO). Although many of these compounds are commercially available in mouth rinse formulations that are unsuitable for children younger than 6 years old, the antiseptics can be delivered in other modes such as gels and varnishes which facilitate better dosage control. These antiseptics were all selected for testing in commercially available mouth rinse formulations without alcohol. The only exception was CPC which could only be purchased in an alcohol containing rinse. The pure antiseptic compounds were then compared in a dose response experiment to determine the minimum concentration at which bacterial growth inhibition occurs.

CHX is a potent bisguanide that inhibits plaque formation, induces cell death and, similar to fluoride, comes in numerous formulations, all of which provide superior substantivity for long-term action at the tooth's surface. Side effects such as staining and taste alteration are common so prescription is limited. Commercially available mouth rinse formulations come in concentrations of 0.12% and 0.2% with and without alcohol. Research has clinically examined toothpastes, gels and mouth rinses containing CHX at concentrations ranging from 0.12% to 2%.[1, 29-31] No studies have performed an *in vitro* dose response examination on CHX and its growth inhibitory effect on cariogenic bacteria.

PI is not commonly used as an oral antiseptic in Australia but provides a valuable alternative to CHX. It is frequently used as an antiseptic in medicine and dental surgery and is bactericidal against mutans streptococci at low concentrations and short application times.[32-34] *In vitro* dose response experimentation is needed for PI.

CPC is a common oral antiseptic with bactericidal tendencies. It has an inherently strong ability to adhere to oral surfaces and bacterial cell walls, though with limited substantivity.[35-37] Similar to CHX, CPC can cause extrinsic tooth staining.[38] Many clinical studies contest to its ability to reduce plaque bacterial levels, however, laboratory based studies are lacking.[25, 35, 37]

EO are found in the formulation known as Listerine. Many studies have been performed on Listerine producing mixed results regarding its ability to reduce plaque bacterial counts.[22, 39-42] However, examination *in vitro* of its specific growth inhibitory effects is lacking.

For safety, low concentrations of antiseptics that can be effectively combined with fluoride compounds would be ideal for paediatric use. The advantages of combinations with synergistic or additive effects are the enhancement of anti-caries and anti-plaque activities of low concentrations of the individual compounds, and the reduction of risks and side effects. Thus far, it is more common to prescribe children at high caries risk two separate products, such as a separate fluoride toothpaste and mouth rinse or additional in-office topical application of another agent such as CHX. This enables control of the concentration and quantity of product applied. However, minimal evidence exists for other combinations of CPC, PI, CHX or sodium hypochlorite (NaOCl). These compounds, along with NaF, were hence tested in all combinations. Additionally, we found SLS to have growth inhibitory effects against *S. mutans* so this was also included as a test compound.

The first stage of this research was to conduct a thorough review of the literature. This established what knowledge was already available on the role of topical chemotherapeutic agents on caries incidence and risk, as outlined above. Gaps in the research surrounding these chemotherapeutic agents were identified, some of which are hoped to be examined further through this research. One such area requiring further research is the growth inhibitory effects of various concentrations of antiseptic agents on cariogenic and non-cariogenic bacteria. Many studies have been performed on the *in vivo* effects of various concentrations of toothpastes or mouth rinses already on the market as detailed below in the literature review. However, the dose response relationship of the active components on bacterial growth inhibition has not been tested. This research plans to analyse these agents using validated agar diffusion techniques in order to determine the bacterial growth inhibitory effects of individual antiseptic and fluoride compounds.

The method chosen to examine these antiseptic agents was the agar diffusion assay. This method is well established and there is much data supporting its use in testing bacterial growth inhibition by antibiotics.[43-45] Following experimentation to establish the techniques and conditions to achieve optimal results, protocol was established to enable the testing of both pastes and solutions.

The bacteria chosen to test were *S. mutans, Streptococcus sanguinis* and *Lactobacillus acidophilus. S. mutans* is commonly isolated from the mouths of children with caries and it has been shown that children who develop caries at a younger age are colonised with *S. mutans* at a younger age with higher bacterial counts.[46-48] Lactobacillus (LB) is involved in the progression of carious lesions.[46] Both *S. mutans* and LB are acidogenic and aciduric and are commonly isolated from carious lesions and plaque in high caries risk individuals.[46] *L. acidophilus* was selected as a representative of the LB group due to ease of purchase. *S. sanguinis,* by comparison, is not associated with caries.[49] In fact, much evidence exists for its antagonistic effect with *S. mutans* whereby if one bacteria is present, the other is

usually absent or low in numbers.[50-52] Thus, *S. sanguinis* was selected for a non-cariogenic bacterial comparison.

This thesis begins with a critical review of the literature on the anti-caries and anti-bacterial effects of toothpastes and antiseptics. This is followed by Chapter Three which is a scientific paper exploring the effects of commercially available toothpastes and their constituents on the growth of oral bacteria using the agar diffusion assay. Chapter Four is a scientific paper reporting on the growth inhibitory effects of mouth rinses and their constituents on oral bacteria. Chapter Five presents the final scientific paper that discusses the effects of combinations of antiseptic compounds and sodium fluoride on oral bacteria. This thesis ends with Chapter Six which contains a general discussion of the research findings, conclusions of the thesis and recommendations for future research.

2. Aims and hypotheses

The aims of the present thesis are:

(1) To compare the anti-bacterial activity of therapeutic agents which have potential for clinical use against *S. mutans* and non-mutans bacteria (*S. sanguinis* and *L. acidophilus).* The agents to be tested include:

(i) Oral antiseptics: chlorhexidine, povidone iodine, cetyl pyridinium chloride, sodium hypochlorite and essential oils;

(ii) Remineralising agents: fluoride compounds, eg sodium fluoride, sodium monofluorophosphate and stannous fluoride;

(2) To investigate combinations of therapeutic agents to determine possible interactions of the agents, e.g. synergistic activity against *S. mutans* and non-mutans bacteria (*S. sanguinis* and *L. acidophilus)*

The aims are based on the following hypotheses:

(1) Some oral therapeutic agents have greater anti-bacterial activity against *S. mutans* compared to non-mutans bacteria such as lactobacilli and *S. sanguinis*

(2) Bactericidal agents such as chlorhexidine and povidone iodine have greater activity against *S. mutans* compared to remineralising agents including fluoride compounds

(3) Bactericidal mouthwashes containing chlorhexidine and povidone iodine have greater activity against *S. mutans* compared to remineralising mouthwashes containing sodium fluoride

(4) Certain combinations of bactericidal and remineralising agents have synergistic activity against *S. mutans*

3. Significance of aims

It is hoped that the results from this study will provide a deeper understanding into the relationship between these oral therapeutic agents and oral bacterial growth inhibition. It is desirable to use a product with high efficacy so that maximal anti-caries effects are achieved at low concentrations. The minimum bacterial growth inhibitory concentrations of the agents identified in this study will aid in the formulation of effective agents at concentrations safe for children that can be delivered in toothpastes, mouth rinses and gels. Furthermore, the results will give insight into which product has the greatest growth inhibitory effects and may be suitable for use in children. Determination of the effects of these agents on both cariogenic and non-cariogenic bacteria will aid in the understanding and design of products that act specifically on cariogenic bacteria without altering the relative proportions of the normal flora.

4. Early Childhood Caries

ECC is a chronic childhood disease that affects the child's well being and quality of life.[3-5, 53] ECC is defined by the American Academy of Paediatric Dentistry as the

presence of one or more decayed (noncavitated or cavitated lesions), missing (due to caries) or filled tooth surfaces in any primary tooth in a child aged 71 months or younger.[2] It has a high prevalence despite being both preventable and reversible.[4] ECC is of great concern due to its rapidly progressing nature and the fact that the child becomes infected with cariogenic bacteria early in the teeth eruption schedule.[4, 54-56]

Caries is a disease process that occurs when cariogenic bacteria such as mutans streptococci (MS), namely *S. mutans* and *Streptococcus sobrinus,* and *lactobacilli* metabolise fermentable carbohydrates.[5] This fermentation process produces acid end products that demineralise the carbonated hydroxyapatite crystals within enamel.[4, 5] Remineralisation naturally occurs when the acids are buffered by salivary enzymes and calcium and phosphate ions re-enter the enamel. Caries arises when the demineralisation-remineralisation equilibrium is unbalanced.[4] Clinically, ECC initially presents with white spots on the maxillary incisors which can swiftly break down into yellow-brown cavities.[57]

The deciduous dentition has inherent morphological differences to the permanent dentition such as being less mineralised and having a greater diffusion coefficient.[58, 59] Deciduous enamel is also half as thick as permanent enamel with narrower enamel prisms.[60] Studies have shown a higher susceptibility of the deciduous dentition to tooth wear which has been attributed to the reduced thickness of the enamel rather than to its greater solubility in acid.[61] As a result of these factors, caries progresses quicker through deciduous enamel.[62, 63]

It has been shown by Isaksson[12] that childhood caries experience has an impact on caries prevalence at age 20 with greater number of carious teeth from ages 3 to 15, resulting in greater number of carious and restored teeth in 20 year olds. Similarly, those with carious lesions in early childhood but are caries-free at age 15, had low caries prevalence at age 20. This is further corroborated by a study by Kohler[64] that showed that children colonised with MS at a young age, had higher salivary MS

levels at 19 years of age and a higher caries experience in terms of decayed and filled surfaces. Those who were non-colonised with MS at a young age were able to remain negative for MS into adulthood. These studies show that controlling caries during childhood can result in reduced caries experience in adulthood.

As such, early preventive dental visits are recommended to ensure early diagnosis and control of risk factors. A systematic review by Bhaskar[65] found early preventive dental visits before the age of 3 to be beneficial for high risk children or those already with ECC. However, there was no evidence for the first visit occurring prior to 1 year of age, despite the assumption that the earlier risk factors are identified, the reduced the caries experience and related restorative expenditures. Following identification of risk factors, primary prevention of caries is necessary through measures such as use of a fluoridated toothpaste, topical application of fluoride or an antiseptic such as PI, fissure sealants on six year old molars and regular dental visits.[66]

4.1 Caries outcomes

Dental caries adversely affects the quality of life of a child and their family. The greatest impact is due to dental pain. This can worsen sleep patterns, the child's ability to eat, ability to learn and overall emotional happiness.[9, 67] A broken down dentition can also lead to teasing in a social situation and embarrassment for the child.[68, 69]

Dental pain is a leading paediatric hospital admission symptom attributable to lack of primary dental care and financial limitations.[6, 70] Facial cellulitis is a common presentation in many of these cases requiring extended hospital stays and extensive dental treatment under a general anaesthetic, accruing large costs.[6] This places huge economic burden on the tax payers and hospitals in the public health system. By comparison, families through the private health system are left with this hefty bill. Also of concern are the waiting lists for treatment within the public health system. The opportunity to have treatment under a general anaesthetic is not common and the family may be left to manage the child's pain for many months until it is their turn for treatment.

Parents generally report negative impacts on physical, mental and social functioning with improvement following dental treatment.[8, 68, 71] Physically, children with ECC are more likely to suffer malnourishment problems such as low body weight, poor height and iron deficiency.[9, 72, 73] Acs and colleagues identified a link between dental caries and failure to thrive in children from low-income families.[10, 11] A review of the literature by Schroth et al[9] also identified speech difficulties either related to speaking or learning to speak as a poor outcome of ECC resulting from premature loss of primary maxillary incisors. However, there is conflicting evidence for these findings as some children adapt very easily. In terms of education, dental pain causes a distraction from learning, poor school performance and commonly results in lack of school attendance whether due to staying home or missing class to see the school nurse.[6, 74, 75] This reduces their socialising time, also delaying their social progress.

Treatment for ECC has been shown to improve many of these adverse quality of life factors. Post-treatment, an increase in social interaction, smiling and attentiveness in school is generally seen.[9] Acs et al[10] showed comprehensive dental rehabilitation resulted in improved ability to eat and sleep and overall improved health leading to catch-up growth to a healthy weight comparable to their age group. However, it must be remembered that severe cases requiring tooth extractions can result in further hindrances to socialisation such as poor appearance, low self-esteem and shyness.

4.2 Caries risk factors

Caries is a multi-factorial disease involving four major factors: bacteria, fermentable carbohydrates, susceptible host and time.[76-79] It is exacerbated by dietary factors such as bottle use and high frequency and long duration of sugar intake. Prolonged bottle feeding especially around bedtime, poor oral hygiene, drinking non-fluoridated water, large family size and low family income all contribute to the high prevalence of ECC.[2, 76, 77, 80, 81] Children with environmental exposure to tobacco smoke during pregnancy and the first year of life also have an increased caries severity.[82]

Undernourished children have increased risk of caries[83] while low birth weight is not considered a risk factor despite the fact that these children tend to be exposed to more risk factors such as poor oral hygiene and increased sugar intake.[84] The relationship between malnourishment and caries risk has been related to low vitamin D, calcium and albumin levels.[85] A further study by Schroth and coworkers[86] found low maternal prenatal levels of Vitamin D impacted the calcium level of the primary dentition and lead to increased rates of ECC.

MS colonies have been isolated from the oral mucosa of pre-dentate infants most likely through maternal transmission.[4, 5] Wan and coworkers[54] examined risk factors that lead to MS colonisation in six-month old predentate infants. They found the leading factors in caries development to be frequency of sugar intake, breast-feeding, saliva transfer habits and the overall degree of human interaction the child experiences.[54] These findings have been substantiated by other studies.[4, 47, 87] Maternal factors included high *S. mutans* levels, poor oral hygiene, high snacking frequency and low socio-economic status.[54] High salivary MS levels in mothers are associated with higher MS levels and caries incidence in their children.[88] This is further substantiated in a study by Gripp and Schlagenhauf[89] that showed that suppressing MS counts in mothers through professional tooth cleaning and CHX varnish application resulted in significantly lower numbers of MS-colonised infants at 2 years of age. Similarly, Robertson and coworkers[90] showed that CHX varnish application in mothers, resulted in reduced severity of caries although not the prevalence of caries. Another known risk factor is malnutrition of the child which can delay tooth eruption and alter the composition of primary teeth and bone, resulting in weakened tooth structure.[78]

Enamel developmental defects are another predisposing factor for ECC. Enamel defects occur when there is a disturbance to the apposition and mineralisation of enamel during formation. Numerous studies have shown that the presence of enamel defects, such as enamel hypoplasia and opacities, to place a child at increased risk of ECC.[91-93] It must be noted that there is complexity surrounding the

study of young children due to the variable number of teeth present, the growing presence of MS and changes in diet.[94]

Saliva plays a vital role in the clearance and inactivation of cariogenic bacteria in the oral cavity as well as providing protection for tooth structure. Saliva contains antimicrobial components such as agglutinins which promote agglutination of *S. mutans* and clearance from the oral cavity.[95] Adhesion of *S. mutans* to tooth structure is reduced by the action of salivary proteases which break down surface protein antigens.[95] MS are regulated by immunological factors such as salivary IgA and IgG which inhibit adhesion and pathogenesis of *S. mutans*.[95] These immunological factors are regulated by HLA genes. Saliva also contains numerous ions including protein, sodium, chloride and bicarbonate, all of which increase with increased stimulation, for example, from chewing gum.[96] Bicarbonate diffuses into plaque where it neutralises acids to increase pH, thus promoting remineralisation.[4, 96] For remineralisation to occur, there are 5 key chemical components required.[97] These include a sufficiently high salivary pH value, sufficiently high salivary calcium and phosphate levels, the presence of salivary peptides that govern nucleation of hydroxyapatite crystals and the presence of the required organic and inorganic matrix (the mineral-deficient enamel or dentin).

Compromised salivary production, either in quantity or quality, increases the risk of caries. This occurs on a nightly basis when, during sleep, saliva production is greatly reduced and swallowing does not occur preventing clearance from the oral cavity.[98] As such, it is vital to clean the teeth prior to bed to reduce caries risk. It may be of benefit to use an anti-cariogenic product overnight for prolonged effect.
Tooth brushing is effective at reducing caries and periodontal disease.[99] It should first be introduced upon the eruption of the first deciduous tooth and should be continued by the parent and then under close supervision throughout childhood. The early establishment of this practice leads to good oral hygiene behaviour throughout life. School tooth brushing programs are common and studies have shown that supervised tooth brushing at school with a fluoridated toothpaste results in a lower

caries incidence in high-caries risk children.[99-101] However, these programs are often only implemented for short periods when funding and timing allows so a thorough tooth brushing regime needs implementation at home by the parents.

4.3 Ecological plaque hypothesis

The ecological plaque hypothesis is one of numerous theories suggested for the aetiology of dental caries.[80] This theory proposes that there are many different bacterial species present in plaque and that changes in the oral environment lead to a shift in the balance of resident oral microflora.[80] By comparison, the specific plaque hypothesis suggests that only a few bacterial species are responsible for the caries disease process, including MS.[80 3-5, 57, 80, 102, 103] There is increasing recognition that *S. mutans* is one of the most important cariogenic bacteria, and acceptance of the concept that reducing its levels will likely lead to a reduction in caries risk.[104] In addition, timely removal of *S. mutans* from the mouths of young children can also lower caries risk by preventing their colonisation of the mouth.[27] MS colonies have been isolated from the oral cavity immediately after birth but increase in numbers with age[55, 81, 103] with a high prevalence in children with a high caries risk.[95] However, it is the intricate relationship between the microbial composition and diffusion properties of plaque rather than the extent of plaque that leads to caries.[80] These factors vary greatly among individuals, sites, and between the primary and secondary dentition and are influenced by disease.[80, 105]

It is well documented that the early presence of MS is a risk factor for ECC however it is simply a marker of a cariogenic environment and not a causal factor.[3, 30, 103] MS grow within the plaque biofilm which provides resistance against antibiotic treatment and the host immune system by creating impenetrable microenvironments.[106, 107] Within these microenvironments, some species are able to thrive while others diminish. For example, MS and lactobacilli act in a positive feedback mechanism supporting the growth of both species.[80] Biofilms containing MS are also part of a positive feedback loop where MS produce competence-stimulating peptides (CSP) that favour cellular accumulation and biofilm formation and these cells, in turn, upregulate CSP production.[108] A resultant increase in the overall numbers and

activity of these and other gram-positive bacteria gives rise to carious lesions.[106]

5. Cariogenic bacteria

5.1 Mutans streptococci (MS)

Caries development has long been documented as the end result of complex interactions between indigenous bacteria.[109] Teeth become colonized by many commensal bacterial species that alter the environment to one optimal for their survival and at equilibrium with host defences.[78] The first oral microbes to colonize the oral cavities of newborn infants are *Streptococcus salivarius*, *Streptococcus mitis* and *Streptococcus oralis*.[95] Two studies have isolated MS and Lactobacillus from the mouths of predentate children with increasing numbers of bacteria with age.[54, 56, 110] Following eruption of the primary dentition, the bacterial profile becomes more complex,[95] however, work by Gizani and coworkers[111] has shown that the streptococcal species colonise soft-tissues at a greater rate than teeth.

MS show a high prevalence in children with a high caries risk.[95, 109, 112] van Ruyven and coworkers[79] determined that as caries status worsened, levels of MS in plaque increased as did numerous other non-mutans streptococci species. *S. mutans* is thought to be associated with the initiation of the disease while *S. sobrinus* is associated with progression[95] since the establishment of *S. mutans* supports the adherence of *S. sobrinus*.[46] However caries experience is higher in children and sites where both bacterial species have been identified.[95, 109, 113] *S. mutans* is more prevalent than *S. sobrinus*, however, *S. sobrinus* has a stronger association with caries incidence.[114-116] For both streptococcal species, caries-free status can be associated with presence of these bacteria.[114, 116, 117] Similarly, caries can occur in the absence of these bacteria.[114, 116, 117] This can be explained by the complex interaction between the heterogeneous microflora, pH, fermentable carbohydrate and tooth factors that result in caries.[114] It has been shown that children who develop caries are colonised by MS at younger ages and have higher MS counts.[48]

Corby and coworkers[106] compared the bacteria present in the oral cavity in health and disease and found MS, Lactobacillus and *Actinomyces* to be abundant in disease.[47] Thus far, no causal relationship has been proven, however, MS has been found to be present in both plaque and saliva in patients with a high caries risk and has a strong, well-documented association to both the onset and development of the caries disease process.[46, 47, 106, 118, 119]

Through the use of denaturing gradient gel electrophoresis, Li and coworkers[120] identified the composition of the microbial plaque community in children with ECC and those that were caries-free. They discovered that plaque in children with ECC has increased numbers of acidogenic and aciduric bacteria but that there is decreased diversity in species. Throughout the caries disease process, different species dominate from initiation through to deep lesion development. Early colonisation with MS is likely to result in their persistence as they have less competition for nutrients and colonisation sites.[95]

5.2 *Streptococcus mutans*

S. mutans is one of the key bacterial species implicated in ECC.[104] Clinical studies have provided evidence that children who develop caries are colonised by *S. mutans* at younger ages and have higher *S. mutans* counts compared to caries-free children.[48] Furthermore, timely removal of *S. mutans* from the mouths of young children can reduce caries risk by preventing their colonisation in the mouth, as demonstrated in studies using CHX varnish,[26] CHX gel,[27] and PI solution.[28]

S. mutans are gram-positive, facultative anaerobes and are non-motile and catalase-negative.[78, 95] Adherence is one of *S. mutans* many virulence factors. *S. mutans* synthesise extracellular polysaccharides in the presence of fermentable carbohydrates which enable adherence to the tooth surface.[46, 47, 95, 121] It involves two stages; initial binding to the acquired salivary pellicle and subsequent sucrose-dependent cellular accumulation.[95] Initially, colonisation is mediated by adhesins produced by *S. mutans*.[95] Later, glucosyltransferases produce extracellular

polysaccharides known as glucans.[95, 114, 121] Glucan binding proteins (GBP) enable strong binding of the bacteria to glucan deposits on the tooth surface resulting in increased plaque thickness, sugar diffusion rates, acid production and thus, cariogenicity.[95, 121] Mattos-Graner and coworkers[121] found a strong relationship between water insoluble glucan synthesis and caries activity in their analysis of 20 MS isolates from 12 to 30 month old children.

S. mutans also synthesise intracellular polysaccharides which are stored and metabolised when dietary carbohydrate resources are low.[47, 95] The outcome is continual acid production especially evident overnight when salivary secretions and sugar consumption are low.

Acidogenicity and acidurance allow *S. mutans* to survive at low pH levels.[46, 47, 95, 119, 121] It is this property that allows them to become the dominant species while less aciduric species are eliminated. They produce acids that reduce the pH to below enamel's critical pH of 5.5 leading to demineralisation, making them a highly cariogenic bacteria.[46, 47, 118, 119] *S. mutans* acidogenicity and acidurance is superior to other plaque microbiota with a terminal pH of approximately 4.[79] *S. mutans* also has the ability to produce antibiotics, namely mutacins, which inhibit the growth of less caries-inductive strains by inhibiting enzyme functions and the generation of adenosine triphosphate, a key energy source.[78]

5.3 *Lactobacillus*

Lactobacillus (LB) is another aciduric and acidogenic, gram-positive microbe that is selected for in low pH environments and, hence, associated with caries progression.[114, 122-124] They coaggregate with *S. mutans* and act as the secondary invader rather than initiator.[123] They consistently produce a terminal pH of 4.2, similar to *S. mutans*.[114] LB are colonisers of mucosal surfaces, especially the dorsum of the tongue, so are present in the oral cavity from an early age, prior to tooth eruption.[46, 123] Colonisation with LB and MS begins at age 6-12months so this is a key time period when prevention should be started.[125] Colonisation with LB has been associated with poor tooth brushing cooperation, unrestored maternal cavitation and

problematic pregnancy.[125] Their presence in saliva occurs due to sloughing of epithelium from the tongue and carious lesions.[46] They are usually found in lower numbers than *S. mutans* and are most prevalent in large caries lesions which extend into dentine.[123, 124] Acid tolerance and acid production are two significant pathogenic properties of LB.[123] However, there is still some uncertainty surrounding the specific mechanisms of these properties.[123]

5.4 Other cariogenic species

Other bacterial species that are currently under scrutiny regarding their cariogenic potential include *Scardovia wiggsiae*, *S. salivarius* and *S. mitis*.[117, 126] Tanner and coworkers[117] has recently identified *S. mutans* and *S. Wiggsiae* as the most frequently caries-associated species in children. Their work found 80% of the children with both these species had ECC while 80% of the children with neither were caries-free. They also isolated *S. wiggsiae* from caries sites independent of *S. mutans* indicating that *S. mutans* may not be necessary for caries initiation and progression.

5.5 *Streptococcus sanguinis*

S. sanguinis is generally considered non-cariogenic[49, 51, 127, 128] despite sharing structural properties and virulence factors with *S. mutans.*[128-130] These virulence factors include attachment to tooth structure through glucan synthesis and production of acids.[128, 129] Adherence to smooth surfaces, however, is far weaker by *S. sanguinis* than *S. mutans* due to inferior glucan synthesis and the requirement for specific conditions for sucrose-dependent adherence.[131]

Westergren and Emilson[49] infected hamsters with *S. sanguinis* and *S. mutans* and found, with similar levels of infection, *S. sanguinis* did not produce caries while *S. mutans* resulted in deep carious lesions. It has been shown that various strains of *S. sanguinis* produce less fissure plaque than *S. mutans* with a reduced extracellular matrix surrounding cells.[127]

A longitudinal study by Caufield and coworkers[50] examined the transmission and acquisition of *S. sanguinis* in mother-child pairs. They found a discrete window of infectivity at 9 months old with increasing proportions of salivary *S. sanguinis* with further tooth eruption. Antagonism between *S. sanguinis* and *S. mutans* was noted as early colonization of *S. sanguinis* delayed colonization of *S. mutans.* Similarly, following colonization with *S. mutans, S. sanguinis* levels decreased. This finding is supported by previous studies that concluded that an increase in the *S. mutans* to *S. sanguinis* ratio increases the risk of caries.[51, 52]

6. Fluoride

There is increasing evidence that good oral hygiene and plaque control is associated with reduced risk for ECC.[87, 132] As most children brush their teeth with toothpaste, it is well accepted that caries prevention from tooth brushing results from the fluoride present in the toothpaste. The role of a good antimicrobial agent is to prevent attachment of bacteria to biofilms and to reduce bacterial proliferation and pathogenicity.[133] Fluoride compounds have been widely studied but it is only since the 1950's that dentifrices containing fluoride have been formulated with therapeutic benefit.[13] For an efficacious dentifrice to be formulated, it is vital that the abrasive system is compatible with the free fluoride ion to produce a bioavailable source of fluoride.[13] Stephen[13] performed a meta-analysis on numerous fluoride compounds and determined that NaF in mouth rinse formulations brings about the greatest reduction in number of decayed, missing or filled tooth surfaces (DMFS) when compared to SnF_2, MFP, amine fluoride (AmF) and acid phosphate fluoride (APF). He also stated that there is a strong dose dependent relationship between fluoride use and caries reduction.[13] Similarly, fluoride has shown a consistent benefit in reducing the progression and incidence of non-cavitated carious lesions.[134, 135]

Primary teeth have a thinner enamel layer, a lower mineral content (including calcium) and higher organic content like that of dentine which makes them more susceptible to cariogenic bacteria compared to permanent teeth.[136, 137] Newly

erupted permanent teeth are also highly susceptible to caries due to their immature enamel.[27, 138] Remineralisation is best promoted by extended availability of fluoride at the tooth surface rather than with higher fluoride dosage.[136] It is also dependent on the bioavailability of the fluoride, the adherence of the compound to the tooth surface and the solubility of the compound.[136]

Commonly in high risk individuals, a combination of preventive products is prescribed and show benefits for combating caries. For example, use of a fluoride mouth rinse as an adjunct to regular twice daily use of fluoride toothpaste is a common suggestion to older children of high caries risk. A meta-analysis of 34 studies relating to fluoride mouth rinses has shown a clear reduction in caries in terms of decayed, missing and filled tooth surfaces in response to use of a fluoride mouth rinse and toothpaste.[139] This is supported by a study by Kaneko and coworkers[140] that found a reduction in MS levels and overall caries experience in schoolchildren following the long-term use of a fluoride mouth rinse. The long-term use of a fluoride mouth rinse has also been shown to have greater action at reducing levels of MS than lactobacilli.[141]

6.1 Fluoride mode of action

Fluoride has numerous modes of action both at a mineral and bacterial level. Fluoride penetrates the tooth structure through the formation of fluorapatite and fluorhydroxyapatite crystals during the remineralisation process.[14, 138, 142] These crystals provide both anti-caries properties by being more acid resistant and anti-bacterial properties by inhibiting bacterial metabolism within the biofilm.[14, 138] The critical pH for dissolution of tooth structure is 5.5.[14] In the presence of fluoride, this value is decreased to approximately 4.4 which allows for prolonged ion exchange at the tooth's surface before demineralisation takes place.[14] Fluoride also enhances calcium and phosphate ion precipitation within the lesion which obstructs further damage by acid penetration and diffusion into the enamel.[14, 138] Simultaneously, calcium fluoride is deposited on the tooth surface and serves as a fluoride reservoir.[143] During remineralisation, part of the calcium fluoride can be redeposited as fluorapatite.[143] These mechanisms aid in maintaining a balance between

demineralisation and remineralisation, favouring remineralisation and preventing caries.

The greatest anti-caries effects are seen when fluoride is maintained within plaque rather than within the tooth itself, where it can exert direct anti-bacterial effects.[142] Fluoride within plaque fluid also has greater remineralisation capacity than that in saliva resulting in reduced enamel solubility.[16, 144] Lynch and coworkers[144] determined that at low pH which usually supports demineralisation, fluoride within plaque can promote remineralisation of the superficial enamel. Secondary demineralisation occurred in the deep areas of lesions while insoluble fluoridated mineral formed within the body and superficial layer. These two steps occur concurrently in the process known as lamination. The deep demineralisation that occurred may be the result of this structure being more soluble than the previously remineralised body of the lesion and also due to the inability for fluoride to reach this depth.

Fluoride is a strong anti-bacterial agent that can both directly kill bacteria and inhibit metabolic pathways.[14] Fluoride is deposited near the surface of plaque where cariogenic bacteria are most metabolically active.[142] Through the binding of electronegative fluoride to positively charged enzymes, fluoride can inhibit their functions and modulate bacterial metabolism.[14] For example, fluoride inactivates the glycolytic enzyme enolase which in turn directly blocks peroxidises.[142, 145-147] This process is enhanced by the presence of magnesium and phosphate and results in inhibition of glycolysis. The divalent cations magnesium and calcium have been shown to increase fluoride binding to bacterial cells both in vitro[148, 149] and in vivo.[150] Clinically, this was evident through an increased fluoride concentrations in plaque and inhibition of bacterial acid production. This is further corroborated by Takahashi and Washio,[151] who showed inhibition of lactate production and a decrease in phosphoenolpyruvate following fluoride use in vivo and by Hannig and coworkers,[152] who showed inhibition of initial biofilm formation on dental hard tissues, especially to dentin. Fluoride can also bind to heme-based catalase such as peroxidase thus

inhibiting its actions directly.[142, 145] This can compromise the bacteria's ability to cope with oxidative damage from hydrogen peroxide in acid environments.[142] Another mode of action is the formation of metal-fluoride complexes impeding the action of proton-translocating F-ATPases, thus preventing ATP synthesis, a key energy source.[142, 145] The most efficacious metabolic regulators are metal complexes between fluoride and aluminium or beryllium.[142] As a weak acid, fluoride enhances membrane permeability to protons thus provoking cytoplasmic acidification and reduced glycolytic enzyme action.[142, 145] It is most effective at arresting glycolysis and thus reducing acidification with lactic acid in intact cells exposed to acidic environments.[14, 145]

6.2 Fluoride formulations and compounds

Fluoride is readily available in numerous formulations and as one of several compounds. So far, NaF has dominated the market in Australia as the anti-bacterial agent of choice in toothpastes, available in both children's low strength fluoride toothpastes as well as adult strength and high dose fluoride pastes. MFP also appears in children's toothpastes while SnF_2 is a desensitizing agent found in some adult strength toothpastes. There are other fluoride compounds utilised in dental products such as AmF and APF, however these are less common. In high risk individuals, a combination of preventive products is prescribed and show benefits for combating caries. For example, fluoride is also available in mouth rinse, varnish and gel formulations recommended for use additional to brushing twice daily with a fluoride toothpaste.

Water fluoridation is a public health caries preventive measure in action in Australia. Reduction in caries in communities with fluoridated water has been reported as between 15 to 60 percent depending on dentition, population and use of preventive agents.[153] However, there is considerable discussion regarding the benefits of community water fluoridation as most populations in the developed world have regular fluoride exposure. Communities with water fluoridation have experienced caries reducing benefits both topically and systemically, pre- and post-eruptively in permanent and primary teeth.[19, 153, 154] Fluoride's pre-eruptive effect on caries in

permanent teeth has been shown to be greater than the post-eruptive effect,[154-156] and this was especially evident for pit and fissure surfaces.[154, 156] Municipal drinking water supplies in temperate climates contain fluoride at 1 part per million (ppm).[57] In addition to water fluoridation, topical fluoride treatment in children aims to prevent new lesions from developing, slow the progression of existing lesions and to prevent the development of fluorosis.[136] For this purpose, fluoride toothpastes are recommended for use twice daily.[19, 133]

There is strong evidence supporting the use of fluoride toothpastes to reduce the incidence of caries and periodontal disease.[16, 18, 157] Various fluoride compounds have been used in toothpastes including NaF, MFP, AmF and SnF_2. The efficacy of a fluoride dentifrice depends on the concentration of fluoride, the frequency and duration of application and the fluoride compound used.[19, 133] It is the frequency and duration of application that has the greatest anti-caries effect with optimal caries reduction obtained when the fluoride levels are maintained at a low concentration by frequent application.[136, 138] As an example, APF reaches maximum fluoride release and uptake by the oral tissues during the first four minutes of application and hence should be applied for this length of time.[19] Fluoride varnishes adhere to tooth surfaces for prolonged periods of time so have increased exposure and uptake rates for enhanced anti-bacterial and remineralising effects[19] and are highly recommended for individuals at high caries risk with professional applications of fluoride varnish recommended for children under 6 years of age.[20] A review by Beltran-Aguilar and coworkers[143] concluded that fluoride varnishes are an efficacious caries-preventive agent and are easy to apply and safe for children and adults alike.

When looking at the optimal concentration of fluoride in toothpaste, conflicting data exists for use in children. Due to the high risk of fluorosis in children, a lower concentration of fluoride is generally desired. However, data shows that the higher the concentration of fluoride, the greater the caries preventive effect.[16, 18] Fluoridated dentifrices of concentrations under 1000 ppm have shown both positive and negative results in relation to their ability to demonstrate anti-caries effect when compared to

placebo.[16-18, 158] Reduction in the amount of toothpaste used is another common option to reduce the risk of fluorosis and allows the use of a higher concentration fluoridated toothpaste (1000 ppm), which is of particular advantage in high caries risk children. The international standard level recommended is now 1000 ppm for use in both children and adolescents.[17] Although the anti-caries effects of toothpastes containing less than 500 ppm fluoride has not been proven, in Australia, low dose fluoride toothpaste is recommended for children younger than 6 years old to reduce the risk of fluorosis.[15]

There are numerous fluoride compounds available on the market. NaF and MFP are both available in children's toothpastes at low concentrations in Australia.[159] NaF forms a calcium fluoride layer on the tooth's surface which provides a slow releasing ionic exchange.[159, 160] It is a strong remineralising agent as it produces a homogeneous lesion and is able to maintain high salivary fluoride levels post-brushing and post-rinsing.[160] MFP is a covalently bound fluoride compound.[159] It is hydrolysed to free fluoride ions in the oral cavity by phosphatases which interact with bacteria and tooth structure ionically in a similar manner to NaF.

A clinical study by Issa[161] (2004) tested toothpastes containing NaF, MFP and AmF at varying concentrations from 500 ppm F to 1450 ppm F. They concluded that the higher concentration of fluoride produced the greatest salivary fluoride levels post-brushing. AmF 1450 ppm F produced the greatest salivary fluoride levels with NaF 1450 ppm F also efficacious 2 hours after brushing.

SnF_2 is a desensitising agent that acts by occluding dentinal tubules[133] with known anti-bacterial effects against *S. mutans.*[162, 163] The anti-caries and anti-plaque effects of SnF_2 were first reported by Muhler.[164] These anti-caries effects have been further corroborated.[165-167] For example, Woodhouse[168] showed a self-applied topical paste containing 10% SnF_2 to reduce caries in terms of decayed, missing and filled teeth by 19% by comparison to a placebo control in school children aged 12-13 years. Similarly, McConchie and coworkers[169] has shown daily use of a SnF_2 mouth rinse in

school children (mean age of 10 years) to result in significant reductions in the numbers of new caries at both 100ppm and 200ppm concentrations. This is a possible method of caries control for high risk children. However, individual advice must be given by oral health professionals accounting for the needs of that particular child.

A few studies by Schmid and coworkers have demonstrated caries-inhibiting effects of AmF.[170-172] The combination of SnF_2 with AmF in a toothpaste and mouth rinse formulation has shown improvements in plaque and bleeding scores, increased plaque and enamel fluoride content post brushing and a decrease in acid solubility.[173]

APF application produces a high concentration of calcium fluoride-like material on enamel and dentin that can last for up to 7 days post-application.[137] These long-lasting fluoride reservoirs maintain the mineralisation equilibrium. Silver diamine fluoride (SDF) has slow dissolution and great anti-caries effect through the formation of silver phosphate ions.[136] It is a low cost product with easy application and works to prevent both caries and dentinal hypersensitivity.[136] Santos and coworkers[136] determined that Duraphat NaF varnish and SDF had the greatest anti-caries effects when compared to other fluoride varnishes and toothpastes. The group of dentifrices included numerous NaF, APF and SDF compounds. However, the application times varied between the groups with some receiving two applications. Similarly, a study by Weintraub and coworkers[174] has shown a decrease in caries incidence following application of Duraphat 5% NaF varnish once or twice per year on school children. On the other hand, a study by Zickert and Emilson[175] has shown topical application of a NaF varnish to not alter the plaque and salivary levels of *S. mutans* applied to school children, suggesting that the caries-reducing effect of NaF varnishes is not due to anti-mutans activity of the fluoride.

Guggenheim and coworkers[105] compared the caries and plaque inhibiting effects of fluoride containing dentifrices and CHX in a rat model. The inorganic fluoride

compounds (NaF and MFP) as well as the organic fluoride compound (AmF) were both effective in caries inhibition when applied topically. Plaque inhibition was found to be highly dependent on the sucrose content of the diet as more agents are able to inhibit plaque formation or reduce plaque extent in a lower sucrose environment. It must be noted that some fluoride compounds commonly known to cause potent caries or plaque inhibition have reduced efficacy in the presence of certain co-agents within the dentifrice. For example, Meridol (GABA International AG, Therwil, Switzerland), an anti-plaque agent containing AmF and SnF_2, shows poor plaque inhibition despite the two compounds being individually strong plaque inhibitors.

Arnold and coworkers[160] compared the anti-caries effects of AmF, NaF and sodium MFP toothpaste formulations. Following demineralisation of 90 caries-free premolars and application of the toothpaste formulations, it was found that complete remineralisation resulted from AmF and MFP compounds while NaF resulted in partial remineralisation with a non-homogenous lesion under polarised light microscopy. AmF left the greatest fluoride content in the superficial layer of the lesion thus making the lesion more acid resistant and stable. Another study by Arnold and coworkers[176] proved that AmF has a strong remineralisation effect when acidified at the optimal pH of 4.5-5.1. AmF application results in fluorapatite and calcium fluoride formation which provide a fluoride reservoir that is pH dependent with greatest remineralisation capacity and reduced solubility at low pH.

Ekambaram and coworkers[158] evaluated the remineralising potential of four fluoride compounds on primary teeth. Following demineralisation, primary teeth were treated with either 500 ppm AmF, 500ppm MFP, 500 ppm MFP and xylitol, 500 ppm NaF or a non-fluoridated dentifrice. NaF was the only compound to decrease the lesion depth while MFP and AmF decelerated the progression of demineralisation.

There is much data supporting the anti-caries effect of MFP.[177-180] However, conflicting studies exist comparing the anti-caries efficacy of dentifrices containing NaF and MFP. One study by Edlund and Koch[181] showed NaF to be more effective

at reducing caries incidence in children when compared to MFP. A meta-analysis by Stookey and coworkers[182] supported this finding that NaF is significantly more effective at preventing caries by comparison to MFP but the authors noted that the formulation and abrasive system used in the dentifrice must provide stability, availability and bioavailability of the ionic fluoride. However, DePaola and coworkers[183] showed MFP and NaF containing toothpastes to have comparable anti-caries effects. A review of 10 clinical studies comparing the anti-caries efficacy of these two common fluoride compounds reported mixed results, depending on the study and the diverse experimental conditions.[184]

A randomized controlled trial by Hausen and coworkers[185] explored the effects of non-invasive caries prevention in children with active initial lesions. The authors employed home fluoride toothpaste use, fluoride and xylitol lozenges, fluoride/CHX varnish, oral hygiene and dietary counselling in the experimental group of 250 children of mean age 11 years old. By comparison to the control group who underwent a basic preventive regime offered by the public dental system, the mean DMFS increments for the experimental group was reduced by 44%. They concluded that caries can be controlled with multiple preventive measures emphasising regular fluoride application.

It is clear that there are mixed results supporting the use of these fluoride compounds. So far, NaF has taken over the market in Australia as the anti-bacterial agent in toothpastes. It is the complex interaction of the ingredients within toothpastes including the fluoride compound, abrasives, binders, surfactants and humectants that result in a viable dentifrice.

6.3 Fluorosis

Dental fluorosis is the hypomineralisation of tooth enamel caused by the ingestion of fluoride above the optimal level during enamel formation.[18] Clinical presentation of fluorosis varies from faint white lines or mottling in its milder form to brown staining or pitting with breakdown of the enamel in its more severe form.[18] The exact

mechanism behind the development of fluorosis is not well understood but it is commonly accepted that the ingested fluoride interferes with enamel formation.[18] There is strong evidence that the use of fluoridated toothpaste can prevent dental caries.[16, 18] In saying that, evidence also supports the statement that the use of fluoridated toothpaste on children under 12 months of age increases the risk of fluorosis.[18] Data is limited due to fewer studies addressing caries in infants and the inability to perform a randomised controlled trial to assess fluorosis itself for ethical reasons. Stronger evidence exists for the relationship between fluoride concentration and fluorosis incidence with higher levels of fluoride (>1000 ppm) putting a child at increased risk.[18] Do and coworkers[186] have shown that fluoride exposure in the first 3 years of life was associated with a higher prevalence of mild fluorosis but significantly lower prevalence and severity of caries. However, the benefits and risks must be carefully examined on a case to case basis as some high-caries-risk children may receive greater health benefits from the use of a topical fluoride agent of higher fluoride concentration.

With a reported increase in dental fluorosis,[187] parents are becoming increasingly wary of this risk following use and ingestion of fluoridated water and toothpastes and, as such, are turning to non-fluoridated options.[187, 188] However, very few non-fluoridated toothpastes have been found to be successful anti-cariogenic agents. Carvalho and coworkers[188] performed agar diffusion assays of 5 non-fluoridated toothpastes against cariogenic bacteria including MS and Lactobacillus. They only found one toothpaste to have growth inhibitory effects and this was not greater than a fluoridated toothpaste. Fluoride is still the leading agent in caries prevention, so it must be stressed that the safe and supervised use of fluoride in children has far superior anti-caries effect with regular use than other products currently available.[139]

7. Oral Antiseptics

Topical fluoride applied in toothpastes is now well accepted as one of the mainstays of caries prevention and in-office applications of fluoride containing varnishes are

currently recommended for high risk children.[16, 17] In addition to topical fluoride, high risk children are likely to benefit from the additional use of anti-bacterial compounds.[21]

7.1 Chlorhexidine

CHX is an antiseptic agent that inhibits plaque formation by selectively inhibiting growth of gram-positive cariogenic micro-organisms including *S. mutans*[189, 190] and some species of LB.[109] It is important to note that each species of oral bacteria responds differently to the agent. It is a potent, hydrophilic bisguanide that can inhibit development of initial carious lesions by reducing levels of MS in both high and low sucrose environments.[105, 189] CHX contains a cation which binds to negatively charged objects including bacterial cell walls, salivary pellicle, plaque and mucosa accounting for its high substantivity.[27] Despite its substantivity, it cannot eliminate MS completely and requires regular use to maintain low levels of these bacteria.[189] At low concentrations, CHX is bacteriostatic when it interferes with cell wall transportation leading to leakage of intracellular components.[29, 30] At high concentrations, CHX is bactericidal causing precipitation of intracellular cytoplasm and impeding the action of glucosyltransferase thus preventing adhesion of bacteria to the tooth's surface.[29] A meta-analysis by van Rijkom and coworkers[190] revealed a caries reduction of up to 46% following CHX use on permanent teeth in 11-15 year old children.

Numerous studies have shown CHX to have superior bactericidal effects against plaque bacteria than other antiseptic mouthwashes such as those containing essential oils (EO), NaF and cetylpyridinium chloride (CPC).[23, 38, 40, 191] However, one study by van Strijp and coworkers[192] did not show a decrease in acid production of plaque samples or prevention of demineralisation of dentine or enamel following twice daily use of a 0.2% CHX mouth rinse.

7.2 Chlorhexidine formulations

Ranked in order of effectiveness, CHX is available as a varnish, gel, mouth rinse, toothpaste and impregnated dental floss.[189, 193] The effectiveness of CHX is highly

variable and dependent on application method, application frequency, composition, fluoride regime and patient caries risk.[190] Close monitoring is necessary and alterations to prescription may be required according to the individuals needs.[29] Composition of varnish or rinse should be ascertained prior to prescription as CHX is commonly inactivated by other agents in the formulation.[133]

CHX gel is most commonly used in a 1% concentration and can be applied at home with a toothbrush or chair side within a tray.[29] When applied as an intensive treatment with numerous applications per day for 2 to 14 days, the reduction in MS levels can last for up to a month.[29] CHX varnish comes in numerous concentrations with 40% being the most effective long-term therapy against MS.[29] Varnish provides increased substantivity with CHX remaining on soft and hard oral tissues for up to 26 weeks post-treatment.[29] Similar to a gel, MS levels remain lowered after the termination of treatment with a CHX varnish but will eventually return to baseline.[29, 30] The most corroborated CHX therapy involves intensive professional application of a gel within a custom-designed tray for 3 lots of 5 minutes repeated on 2 consecutive days or professional application every 3-4months. Longer application intervals have reduced inhibitory effect.[30, 193]

For use in paediatric dentistry, CHX has been shown to be highly effective in reducing ECC when applied in the form of a high concentration (40%) varnish delivered 6 monthly by professionals.[194] However, clinical trials have showed that daily application of 0.12% CHX gel did not result in lower caries rates compared to controls, probably due to poor compliance of gel use associated with the unacceptable taste of the gel for young children.[195]

According to Twetman,[30] at home, self-application of CHX gel for 5 minutes each day for 14 days can be effective. However, the home use of CHX is commonly ineffective due to the bitter after-taste of CHX resulting in poor compliance.[1, 190] Decreasing the concentration or application time can combat this problem.[29] Further clinical trials were performed by Pukallus and coworkers,[1] where a CHX gel was used daily along

with twice daily tooth brushing with 304% fluoride toothpaste on infants aged 0-24months. They found that the tooth brushing reduced the incidence of caries regardless of the use of CHX but this was attributed to low compliance. Similarly, Emilson and coworkers[196] showed that after 14 days of applying a 1% CHX gel, all 5 participants in the study eliminated *S. mutans* from their mouths.

Common side effects following long-term CHX usage include altered taste sensation, tooth discolouration, gingival bleeding, increased calculus formation and epithelial desquamation.[30] As a cationic antiseptic, it precipitates dietary chromogens to produce extrinsic stains, so is only prescribed for short term use to avoid this reaction.[38] As such, CHX is contraindicated as a primary caries preventive therapy and is only indicated in support of other oral hygiene techniques in patients with active caries or those who are medically compromised or disabled.[30] However, these common complications are not always reported as each individual responds differently to CHX treatment.[27]

The first longitudinal, double-blind, placebo-controlled CHX study on MS colonisation was performed by Wan and coworkers.[31] Their results showed a significant decrease in MS levels following use of a 0.2% CHX gel in children with low initial MS levels (<300 CFU/ml). However, children with high initial MS levels and those taking the placebo, showed similar results over the course of treatment. MS colonisation was also found to be highly dependent on tooth brushing and diet.

Law and Seow[27] later studied MS positive children over a 12 month time period. Along with dietary and oral hygiene advice, a 0.2% CHX gel was brushed once daily for 6-12 months. A decrease in MS levels with longer use of CHX was noted. After 3 months, 28% of the children were negative for MS while after 12 months 70% of the children were negative for MS. When examining post-treatment recolonisation, they found that after 9 months, 20% of the children were still MS negative indicating the good substantivity of CHX. Discontinuation of CHX therapy often results in

maintained caries reduction due to development of good dietary and oral hygiene habits as well as CHX's superior substantivity.[29, 189]

7.3 Povidone iodine

PI is an antiseptic agent used in many areas of medicine and dentistry such as prophylactically prior to, during and after dental surgery.[32-34] It is a complex of iodine with polyvinyl pyrrolidone.[33] This compound decreases side effects of pure iodine such as staining and irritation and increases water solubility for enhanced action.[33] Contraindications include pregnancy, thyroid disease and iodine hypersensitivity.[32] This compound has a high specificity for highly cariogenic bacterial species such as MS and produces bactericidal effects.[33, 34] PI is water soluble with slow release of iodine resulting in long-term anti-bacterial effects.[33] As an oral antiseptic, PI is most commonly available as a mouth rinse.

Work by Lopez and colleagues[24, 28] supports the concept that PI reduces MS levels in children at high risk of ECC. They showed that an in-chair topical application of 10% PI is effective at preventing white spot lesions in toddlers when applied every 2 months, even if the child is bottle feeding on cariogenic substrates at naptime.[24, 28] Similarly, Berkowitz and coworkers[197] have shown that a once-only topical application of 10% PI followed by APF in children produces a prolonged decrease in MS and LB levels over 3 months. This approach did not, however, prevent new carious lesions from developing over the subsequent year post application, probably due to persistent cariogenic dietary factors and poor oral hygiene.[197] PI can have an MS-inhibitory effect of over 95% CFU/ml of saliva.[197] This MS suppression can be maintained in the oral cavity for up to 90 days post-application possibly due to the increased susceptibility of demineralised enamel to PI.[197] Simratvir and coworkers[198] showed that a 3-monthly topical application of 10% PI following dental restorative treatment can reduce *S. mutans* levels and prevent further occurrence of caries.

Tanzer and coworkers[34] found the conditions for optimal bactericidal effects to be similar to those for CHX. They showed highly cariogenic species such as MS to be killed by inorganic iodine at concentrations as low as 0.04% applied for a single 5

minute treatment while less cariogenic species only displayed reduced acid production.[34] This specificity is supported by PI's selective inhibition against glucosyltransferase, a highly active enzyme in cariogenic bacteria.[33, 34]

7.4 Essential oils

EO containing mouthwashes are currently on the market, one of the leading brands being Listerine. Their bactericidal properties can be attributed to their ability to effectively permeate through established plaque.[199] There the EO disrupt the cell wall and inhibit enzyme activity resulting in cell death. They also prevent bacterial aggregation and proliferation.[199] However, there is conflicting data in respect to Listerine's anti-plaque efficacy. Several studies have shown beneficial effects of EO mouth rinses such as significantly reducing total plaque bacterial counts,[22, 41, 200-202] reducing MS levels in teenagers,[203] and improving plaque and bleeding scores in orthodontic patients;[204] while other studies have shown negligible anti-bacterial effects.[39, 40] Specific action against MS was tested and confirmed by Fine and coworkers.[22] They took plaque swabs and saliva samples from subjects using Listerine mouthwash twice daily in addition to regular oral hygiene practices and found a significant reduction in MS levels both from plaque and saliva following a 12-day period, with plaque showing a greater reduction in bacterial counts. When combined with regular tooth brushing, a greater reduction in plaque accumulation was noted, thus indicating that Listerine was more active against newly formed plaque.[191, 205, 206]

By comparison to 0.2% CHX, Netuschil and coworkers[191] found an essential oil mouth rinse (Listerine) to have significantly less reduction in plaque index, total bacterial counts, colony-forming units and plating efficiency. The essential oil did hinder plaque regrowth but results were comparable to the control rinse of 0.02% quinine-hydrochloride. Another study by Wiken Albertsson and coworkers[42] has shown EO to be less effective at reducing the amount of *S. mutans* and LB.

Numerous studies have explored individual EO and their effects on oral bacteria, specifically *S. mutans.* Promising results have been shown for essentials oils

isolated from *Lippia sidoides*[207, 208], *Myristica fragrans* (nutmeg)[209], *Curcuma longa*[210] and cloves[211].

7.5 Cetyl pyridinium chloride

CPC is a strong antiseptic mouth rinse commonly prescribed in the dental field. CPC is a quaternary ammonium compound with strong anti-bacterial properties including its inherently strong ability to adhere to oral surfaces, though with limited substantivity.[35-37, 212] It has high binding affinity for negatively charged bacterial cell walls allowing for membrane disruption, leakage of cytoplasmic components and inhibition of metabolism and proliferation.[23, 25, 37, 213] These behaviours are similar to those seen with CHX, however, clinically CPC produces a relatively quick return to pre-rinse salivary bacterial levels with short residual salivary anti-bacterial activity.[36] It also prevents cellular aggregation and thus plaque maturation.[37] Similar to CHX, CPC can cause extrinsic tooth staining, though to a lesser extent than CHX.[38] A high affinity for gram-positive species such as MS has been shown.[23, 25, 213]

Numerous studies give evidence of CPC's ability to reduce plaque bacterial levels and, hence, plaque formation in both alcohol-containing and alcohol-free formulations.[23, 25, 35, 37, 212, 214, 215] CPC has also been shown to reduce gingival inflammation, gingival bleeding and plaque levels by comparison to a placebo mouth rinse.[216, 217] The use of CPC in gel or varnish form has thus far only been studied by combining CPC with zinc gluconate in a gel [218] or with acrylic resin in a varnish[219] producing anti-calculus and anti-bacterial effects.

In trials employing CPC mouth rinses as adjuncts to regular tooth brushing, the reduction in plaque and gingivitis was limited.[220] The cause has been proposed as an interaction between the CPC and toothpaste ingredients resulting in inactivation of an active ingredient.[220] It has also been suggested that poor compliance in at-home trials and the Hawthorne effect may be involved.[220] A study by Sheen and colleagues[38] has shown a reduction in tooth staining when CPC and CHX were used both immediately before and after a toothpaste slurry, indicating inactivation of the

antiseptic agent. They concluded that these oral antiseptics should be used some time after the use of toothpaste.

By comparison, there is minimal evidence for the use of CPC in children as an anti-caries agent. Work by Pawha and coworkers[221] demonstrated the clinical effectiveness of a commercially available 0.07% CPC mouth rinse at reducing the plaque index and bleeding scores in children and young adults (10-25 years old) undergoing fixed orthodontic appliance treatment. Further, use of a pre-surgical 0.05% CPC mouth rinse by children aged 10-15 years has shown efficacy at reducing both aerobic and anaerobic oral microorganisms.[222]

Numerous comparisons between CPC and various other mouth rinses have been performed. By comparison with a fluoride mouth rinse, CPC has been found to significantly reduce anaerobic bacteria in supragingival plaque over a 14 day period.[223] CPC additionally reduces periodontal and halitosis associated pathogens, however to a lesser extent than a CHX mouth rinse.[215] Nelson-Filho and colleagues[224] showed that a 0.05% CPC mouth rinse is effective at disinfecting toothbrushes used by preschool-aged children at day-care, although not to the extent of CHX. Similar plaque levels and gingival bleeding scores have been observed in trials comparing a CPC mouth rinse to an essential oil mouth rinse.[225, 226]

Patients undergoing fixed appliance therapy have a greater caries risk related to increased levels of *S. mutans.*[227] Al-Musallam[228] has shown the incorporation of CPC into a commercially available orthodontic adhesive (Transbond XT) to have antimicrobial activity with increased bacterial inhibition with increased concentration. With 2.5% CPC in the adhesive material, the tensile strength remained constant.

7.6 Sodium hypochlorite

Sodium hypochlorite (NaOCl) is commonly used as a disinfectant for cleaning baby products such as bottles and pacifiers. Disinfecting objects shared between mother and child, such as bottles, cutlery and toothbrushes using a strong antiseptic can

help to reduce the vertical transmission of oral cariogenic bacteria from mother to child. NaOCl has also been suggested as a disinfectant for toothbrushes, with promising results for MS reduction.[229] NaOCl is not commonly used as a mouth rinse due to its poor palatability. However, recent studies are reviewing its use for treating adult periodontal disease in formulations with improved taste as evidence shows it can improve plaque and bleeding scores at concentrations as low as 0.05%.[230, 231]

7.7 Sugar alcohols

As mentioned previously, caries is initiated by the breakdown of carbohydrates by certain bacteria. As such, it is safe to assume that dietary sugar restriction will likely lead to caries reduction. This assumption has lead to examination of sugar alternatives. The sugar alcohols are a dietary sugar substitute that can be used to reduce the incidence of caries.[78, 232] They are metabolically derived from their corresponding aldose sugars.[232] As such, they are also known as alditols. Sugar alcohols are crystalline substances with varying sweetness.[232] The two most common sugar alcohols used in the dental field are xylitol and sorbitol. One of the anti-cariogenic properties is the reduced ability or inability for oral microorganisms to metabolise sugar alcohols.[97, 232, 233] However, the different molecular masses provide variation in biological properties of alditols. This must be remembered as each alditol has unique properties and differing anti-caries capacity. Many alditols have been tested for fermentation by oral bacteria and have been subdivided as either hypo- or non-acidogenic.[234] Sorbitol is hypo-acidogenic while xylitol is non-acidogenic and anti-cariogenic.

7.7a Xylitol

Xylitol is available in numerous formulations including chewing gum, pastilles, dentifrice, mouth rinse and pacifiers.[232] Use of a xylitol dentifrice provides simultaneous mechanical cleaning and can provide additive effects with other ingredients such as fluoride and detergents. These additive effects can also be achieved with the use of a mouth rinse which is normally a short-term treatment

option. A review by van Loveren[25] concluded that xylitol is generally considered more effective than sorbitol at caries prevention clinically but its anti-mutans effects are not confirmed. Reviews by Mickenautsch and colleagues[235] and Lingstrom and colleagues[233] have produced evidence supporting a reduction in caries following sugar-free gum chewing. However, both reviews show disparate findings for direct therapeutic effects by xylitol or sorbitol on dental plaque. Both concluded that further well-designed randomised trials are needed to confirm the caries-reducing effect of sugar alcohols and their underlying mechanisms.

As mentioned earlier, bacterial colonisation in infants is highly associated with maternal bacterial counts and oral hygiene practices. As such, treatment targeting both mother and child are of use in combating the disease early. A series of studies by Söderling and colleagues[236-238] have shown that reducing maternal MS counts with xylitol chewing gum can significantly reduced MS colonisation in their children's teeth at the age of 2 years and significantly reduced dentinal caries at the age of 5 years. In a follow up study, they showed early prevention of MS transmission from mother to child can have long-term effects on the child's MS colonisation but preventive measures must be maintained over a lifetime as their effects diminish with time. It has been shown that a minimum of 5-6 grams and 3 exposures per day from chewing gum is needed for the clinical reduction of plaque and saliva MS counts.[239-241]

The use of xylitol chewing gum by children has also shown efficacy for reducing plaque mass as well as MS and lactobacilli counts.[242-244] Furthermore, xylitol gum can have a long-term growth retarding effect on MS and lactobacilli as reduced bacterial growth has been observed 15 months following the termination of xylitol use.[244] By comparison, these studies found only a reduction in plaque mass following use of a sorbitol chewing gum possibly due to passive effects by the stimulation of saliva.[242-244]

7.7b Sorbitol

D-Glucitol (Sorbitol) is the most common sugar substitute in the United States for use in chewing gums and over-the-counter medicines. This is owing to its cost-effectiveness by comparison to other sugar alcohols such as xylitol. It is a hexitol-type molecule that is more effective at reducing the incidence of caries when compared to sucrose but is considered inferior to xylitol.[232, 245] Mixtures of xylitol and sorbitol can exhibit additive effects; however the concentration of xylitol in the mixture tends to determine the anti-caries effect.[232, 246]

Sorbitol is a low cariogenic sweetener as it does not lower plaque pH to below the threshold for enamel demineralisation, unlike sucrose.[245] However, sorbitol is not non-cariogenic as oral bacteria can adapt to metabolise sorbitol when their regular sugar source is restricted.[245] Evidence suggests that sorbitol can in fact stimulate the growth of *S. mutans* in such conditions as it is readily converted to its glucose and fructose components which act as the carbohydrate substrates for these bacteria.[232] As such, consuming large amounts of sorbitol can increase growth and fermentation of cariogenic bacteria.[77]

8. Dentifrice Ingredients

Tooth brushing has long been accepted as a mechanism to maintain acceptable oral health with fluoridated toothpaste being the long-term gold standard in caries prevention. In addition to fluoride, dentifrices contain a number of other agents which may inhibit the growth of *S. mutans,* including surfactants, antiseptics such as triclosan, flavours and humectants. Other dentifrice components such as binders, fillers and abrasives are unlikely to exert antibacterial actions. Common agents include sodium pyrophosphate, polyethylene glycol, sodium lauryl sulphate and triclosan. The role of these agents are explained further in this section.

8.1 Sodium pyrophosphate

Sodium pyrophosphate is a tartar control agent found in many toothpastes and has been shown to reduce calculus by at least 20% by comparison to a control, non-pyrophosphate containing dentifrice.[247, 248] Despite this, sodium pyrophosphate is not known to have any anti-bacterial effects. However, through the blocking of active protein sites on apatite crystals, some propose that sodium pyrophosphate can reduce bacterial adhesion to tooth surfaces.[247, 249] By blocking active protein sites such as calcium sites on the hydroxyapatite crystals, sodium pyrophosphate retards pellicle formation and displaces proteins and organic substances from the enamel surface. This results in good cleansing of the oral cavity, reduced calculus formation and possibly reduced bacterial adhesion to the tooth surface.[247, 249, 250] Evidence suggests that sodium pyrophosphate does not influence the remineralisation or anti-caries effect of fluoride[249] and can, in fact, have enhanced anti-calculus effect when combined with NaF.[251]

8.2 Polyethylene Glycol

In the field of medicine, polyethylene glycol (PEG) is widely used as for bowel preparation before colonic surgery and colonoscopy due to its ability to form a physical barrier between the intestinal epithelium and invasive microbes.[252-254] Although interference with bacterial growth is not a known property of PEG, several studies have shown anti-bacterial effects.[255, 256]

In the dental field, PEG is a humectant used in toothpaste. The main anti-bacterial action attributed to PEG is the reduction of bacterial adhesion to enamel surfaces through a high binding affinity for hydroxyapatite crystals.[257-259] A novel polymer of PEG with pyrophosphate was tested by Chen et al and was shown to effectively inhibit salivary protein adsorption to artificial hydroxyapatite and inhibit saliva protein-promoted *S. mutans* adhesion.[259]

Recent research is exploring the treatment of titanium implant surfaces with PEG in order to reduce bacterial adhesion and, hence, biofilm formation on the implant surface aiming to improve success rates of implant therapy.[260, 261] This is achieved due to PEGs ability to inhibit salivary protein adsorption, involved in the initial stages

of biofilm formation, which was shown by Tanaka and colleagues to be more effective on rough implant surfaces rather than on smooth implant surfaces. They also identified a reduction in biofilm formation and easier detachment of *S. mutans* biofilms from the PEG-titanium implant surface. [261] Along with a reduction of bacterial adhesion to the implant surface, Bozzini and coworkers[260] have shown an increase in PEG surface density on the implant to result in increased osteoblast proliferation and resultant stimulation of bone formation.

8.3 Sodium lauryl sulphate

Sodium lauryl sulphate (SLS) is an anionic detergent found in most toothpastes available on the market today. SLS is used for its foaming qualities but also has anti-bacterial actions. It undergoes adsorption to bacterial cell surfaces to interfere with cell integrity.[262-264] SLS inhibits growth of mutans streptococci and inhibits enzymic activities of glucosyltransferases.[265, 266] Glucosyltransferases synthesise water-insoluble glucans from sucrose which is a key step in caries induction.[265] SLS has been shown to reduce lactate production by *S. mutans* and to reduce extracellular polysaccharide formation.[264]

Another mode of action *in vivo* is the alteration of the adsorption of proteins to enamel, thus affecting the formation of the salivary pellicle.[267] A study by Hamilton and coworkers[268] has shown SLS to have etching abilities and to result in an undesirable increased susceptibility of treated enamel to acid attack. Their study, however, used a high concentration of SLS (10%) for a short period of time, unlike tooth brushing and was not clinically based.

Petersen and colleagues[264] showed an additive inhibitory effect when combining SLS with NaF. This was only seen, however, for thinner glucose-grown biofilms. Another study has shown that the presence of SLS in a dentifrice with fluoride or as a pre-treatment prior to fluoride application, can reduce the amount of fluoride deposited on the tooth surface and thus reduce the effects of fluoride.[269] The combining of SLS with zinc citrate has also been researched and shown additive inhibitory effects with a 70% reduction in plaque scores from baseline.[270]

8.4 Triclosan

Triclosan is a broad-spectrum anti-bacterial agent found in numerous toothpastes that is active against both gram positive and gram negative bacteria including *S. mutans.*[271] It's main mode of action involves the disruption of bacterial cytoplasmic membranes to block fatty acid biosynthesis.[272] It has greatest effect on plaque-free surfaces indicating it can prevent bacterial adhesion and inhibit growth of plaque.[271] Two downfalls of triclosan include poor water solubility and poor retention in the oral cavity.[273] As such, it is often combined with a copolymer of polyvinylmethylether/maleic acid (PVM/MA) for enhanced substantivity and efficacy.[272, 274, 275] A copolymer of 0.13% calcium glycerophosphate in a calcium carbonate base has been shown to significantly reduce plaque and gingival bleeding scores by comparison to PVM/MA with triclosan as a constant.[276] Similarly, combining triclosan with zinc citrate has shown additive anti-plaque effects.[271] Gilbert and coworkers[277] reported Triclosan's oral retention measurements two minutes following tooth brushing as 38% with greater retention quantities following the use of a higher dose of Triclosan.

Review articles report clinically significant improvements in plaque control and gingivitis following use of a triclosan-containing dentifrice.[272, 275] However, triclosan has been reported to neither enhance nor interfere with remineralisation or the anti-caries effect of fluoride.[272, 278, 279] Hawley and coworkers[280] concluded that a dentifrice containing 0.24% NaF/silica abrasive with 0.3% triclosan/2.0% copolymer produces equivalent anti-caries effects as a dentifrice without the triclosan/copolymer. A dentifrice containing 0.24% NaF/silica abrasive with 0.3% triclosan/2.0% copolymer has also been shown to produce enhanced remineralisation and enhanced fluoride uptake on interproximal enamel by comparison to a dentifrice without the triclosan/copolymer.[281] Triclosan has been shown to produce enhanced protection for dentin when exposed to low concentrations of *S. mutans,* ie a mild acid attack, as measured by lactate production, calcium loss and mean final pH over five experimental days.[279] A study by van Loveren and colleagues[282] revealed that pre-treatment with a triclosan-

containing dentifrice greatly reduced the cariogenic outcomes on enamel and concluded that enamel may act as a reservoir for triclosan. Numerous review articles report clinically significant improvements in plaque control and gingivitis following use of a triclosan-containing dentifrice.[272, 274, 275, 283]

Another delivery system of recent interest is the use of dentotropic micelles which aim to increase triclosan solubility, increase hydroxyapatite binding and provide prolonged drug release.[273, 284, 285] *In vitro* testing of triclosan-loaded tooth-binding micelles has shown great promise including the ability to inhibit biofilm formation and reduce the viability of pre-existing biofilms.[273] This antimicrobial formulation has also been used with pyrophosphate.[285]

9. Interactions

It is possible that certain preventive agents have synergistic and additive anti-bacterial effects when combined. Synergism can be defined as the interaction of two or more substances to produce a combined effect greater than the sum of their separate effects.[286] As low concentrations of chemicals are required to improve safety and reduce side effects in children, it is pertinent to look for combinations of compounds that have synergistic or additive anti-bacterial effects. As synergistic agents produce a combined effect greater than the sum of their separate effects, efficacy can be achieved using low concentrations of individual compounds that are safe for children.[287] Combinations with additive properties are also beneficial as relatively low concentrations of individual compounds can be employed to boost the effects.[288] These strategies for boosting the efficacy and safety of medications are commonly applied in medicine and dentistry. For example, the staining and mucosal irritation of pure iodine is reduced by combining iodine with polyvinyl pyrrolidone.[33]

In contrast to synergistic and additive effects which are usually beneficial, some combinations of antiseptics can result in interference and reduced efficacy.[289, 290] Drug-drug interactions are common and usually result from the interaction of two

agents which increase or diminish the magnitude or duration of action resulting in an unintended reaction.[289, 290] Chemically reactive agents compete with each other to prevent the other agent from interacting with its intended receptor target or by reacting with molecules, ions or proteins.[289]

9.1 Fluoride and Chlorhexidine

Initial studies have explored the combined use of separate fluoride and CHX gels which have been found to have synergistic anti-bacterial effects with extended MS suppression.[189] Simultaneous application of varnishes, gels or rinses containing CHX or fluoride have been shown to lower *S. mutans* counts and increase enamel mineral density.[291-293] In addition, a varnish containing both NaF and CHX was associated with sustained reduction of interdental *S. mutans.*[294] A blind, randomized-controlled trial by Olympio and coworkers[295] studied 83 orthodontic patients and their use of either a 1100ppm NaF toothpaste, a 0.95% CHX toothpaste or these dentifrices in combination. They confirmed that CHX was superior to NaF in terms of reduced gingivitis, bleeding scores and plaque and calculus formation. Combined treatment showed additive anti-plaque effects. In a few volunteers, staining and bitter taste was observed. The use of a 1% CHX gel in office followed by at home application of a 1% NaF gel for a 2 week period produced a greater reduction in salivary *S. mutans* levels and slower rate of return of *S. mutans* than the placebo group.[296]

Many studies have tested the effects of combining fluoride and CHX in a single product. It has been found that these two agents can co-exist without interfering with the other's protective properties.[297] As a dentifrice, fluoride and CHX combinations have been shown to produce a greater reduction in caries than a single fluoride or CHX dentifrice.[298] However, the difference was not statistically significant. A two year study by Luoma and colleagues[293] observed a group of high caries risk 11-15 year olds provided with either a fluoride mouth rinse, a combined fluoride-CHX mouth rinse, a placebo solution or no rinse. The reduction in mean DMFS increments and bleeding gingival scores was greatest for the combined fluoride-CHX mouth rinse group with fluoride and the placebo rinse having comparable results. A similar study by Spets-Happonen and coworkers[299] showed a reduction in S. mutans levels and

gingival bleeding scores following daily rinsing with a CHX-fluoride rinse. An *in vitro* study by Meurman[300] confirmed synergistic interactions between CHX and fluoride in relation to their ability to inhibit adhesion of bacteria to enamel and their anti-bacterial effects. The combined CHX and fluoride group showed the greatest reduction in adsorption of radiolabelled bacteria to hydroxyapatite and functioned at the lowest inhibitory concentration by comparison to fluoride and CHX alone. It was also able to cause disruption to streptococcal cells similar to the CHX group.

9.2 Fluoride and Povidone iodine

Numerous studies have explored the combined use of a fluoride varnish and topical PI treatment showing additive anti-caries effects. The combined use of topical PI and fluoride varnish has been shown to result in more caries-free first permanent molars in children aged 5 to 7 years within a school year compared to fluoride varnish alone.[288] It was concluded that the combined topical application of this antiseptic agent at the same time as fluoride varnish provides additional caries preventive effects to a fluoride varnish alone. Milgrom and colleagues[301] performed a further study showing a 31% reduction in new carious lesions over fluoride varnish alone in the primary dentition following the combined treatment with separate 10% PI and 5% fluoride varnishes. A clinical trial by Xu and coworkers[302] revealed no added bacteriostatic or cariostatic effects following use of a single PI/fluoride foam rather than regular fluoride foam and concluded that further evidence is required to support this relationship.

9.3 Fluoride and Essential oils

EO in combination with fluoride have been evaluated in several studies with mixed results. An EO and fluoride mouth rinse has been shown to produce greater fluoride uptake and enamel remineralisation than a separate fluoride or EO mouth rinse as determined by surface microhardness evaluation.[303] However, another study testing the microhardness of demineralised enamel in a similar modality revealed only an equal increase in microhardness following treatment with an EO/fluoride mouth rinse and a fluoride rinse alone.[304] Plaque acidogenicity has also been shown to be affected by an EO mouth rinse, with no added benefit from the addition of fluoride.[305]

Zheng and Wang[306] performed a comparison between a CHX rinse, an EO and EO/fluoride mouth rinse on root-caries pathogens (*S. mutans, S. sobrinus, Lactobacillus rhamnosus* and *Actinomyces naeslundii)* and found that CHX had the greatest effect on bacterial growth inhibition while the EO/fluoride rinse could not effectively reduce numbers of these bacteria.

9.4 Fluoride and Sodium lauryl sulphate

SLS has long been used as a detergent in fluoridated toothpastes. An early study by Barkvoll and coworkers,[269] explored the effect of SLS on the deposition of calcium fluoride on enamel *in vitro*. They found that the use of a dentifrice containing MFP and SLS as well as a pre-treatment of SLS before fluoride application, decreased the amount of calcium fluoride deposited on enamel. This was believed to prevent fluoride's extended anti-caries effect at the tooth surface. Giertsen[307] examined the effect of mouth rinses containing various combinations of triclosan, zinc, SLS and fluoride on acid formation by dental plaque *in vivo*. Her results revealed a slight but statistically insignificant decrease in acid formation as tested by pH levels following use of a mouth rinse containing fluoride and SLS. This is further supported by Petersen and coworkers,[264] who examined the effects of NaF and SLS on acid- and polyccharide-formation of biofilm and planktonic *S. mutans* cells. They found an additive inhibitory effect on *S. mutans* acid formation and extracellular polysaccharide formation which can enhance the inhibition of dental plaque formation and pathogenicity.

9.5 Fluoride and Cetyl pyridinium chloride

CPC is an anti-plaque compound whose anti-bacterial effects are thought to augment the effects of fluoride toothpaste.[308] An experimental rinse containing both CPC and NaF, used twice daily after regular tooth brushing over a period of 2 months demonstrated an improvement in plaque scores and dental sensitivity.[309] Another study showed that tooth brushing and rinsing with CPC maximised plaque reduction.[308] In contrast, other studies have shown that the combined use of CPC and fluoride toothpaste have antagonistic effects in terms of plaque scores.[38, 220] There is evidence for the inactivation of CPC by fluoride, similar to CHX, so it is

suggested that at least 30 minutes be allowed between brushing with a fluoridated dentifrice and rinsing with CPC to optimise the anti-plaque effect.[38, 220, 308]

9.6 Chlorhexidine and Sodium hypochlorite

The combined use of NaOCl and CHX has predominately been within the field of endodontic irrigants. Their anti-caries effects have not been well researched. The combined use of NaOCl and CHX as irrigants results in precipitate formation which occludes dentinal tubules, reduces marginal sealing and favours microleakage.[310-312] It is believed that the precipitate is parachloroaniline, a hydrolysis product of CHX with known toxic and carcinogenic degradation products.[311, 313] This has been evaluated through many methods including x-ray photon spectroscopy[311] and environmental scanning electron microscopy.[313] These changes were also shown to be dose dependent, both in respect to the concentration of CHX and the concentration of NaOCl.[310, 311] Basrani and coworkers[311] showed that a minimum concentration of 0.023% NaOCl will result in precipitate formation and colour change when added to 2% CHX. It is, hence, suggested that NaOCl and CHX not be used simultaneously during endodontic procedures.[310, 311] Valera and coworkers[314] prepared root canals irrigating with 2.5% NaOCl, then saline solution, then CHX gel which resulted in greater cleaning of root canal walls with the highest number of open dentinal tubules by comparison to a CHX liquid. Use of EDTA as a final irrigant also showed improvement in cleaning. Vianna and Gomes[315] performed an agar diffusion assay and broth dilution test on the combined growth inhibitory effects of NaOCl and CHX at varying concentrations on *Enterococcus faecalis*. They concluded that the combination of NaOCl with CHX did not produce additive effects greater than that of CHX alone, and, in fact, the higher the concentration of NaOCl, the less growth inhibition of *Enterococcus faecalis*.

9.7 Chlorhexidine and Povidone iodine

The combined efficacy of CHX with PI in the dental field has not been investigated. Most research into the combined use of CHX and PI is in the field of surgical site antisepsis. Many articles have indicated that the combined use of both agents in surgical site preparation results in synergistic anti-bacterial effects and decreased

rates of infection.[316-318] Guzel and colleagues[316] recommended a three minute cleaning with CHX followed by a 30 second cleaning with PI for improved skin disinfection in preparation for neurosurgical procedures. This antiseptic combination has also been suggested for decontamination of human donor eyes prior to corneal preservation.[319]

9.8 Chlorhexidine and Sodium lauryl sulphate

Little research has explored the interaction of CHX with SLS in a single product. It is well known that toothpastes containing SLS can reduce the antimicrobial effects and substantivity of CHX due to formation of a salt following a cationic (CHX) and anionic (SLS) reaction.[320-325] Work by van Strydonck and coworkers[326] showed that rinsing with a 1.5% SLS slurry prior to use of a 0.2% CHX rinse reduced the anti-plaque efficacy of CHX. This has been confirmed by a systematic review by Kolahi and Soolari[320] which revealed that CHX interacts with SLS to produce insoluble salts which reduce the anti-plaque effect of CHX. Using an SLS containing mouth rinse or a dentifrice slurry following a CHX rinse significantly increased plaque scores. Inactivation of CHX was further examined by Kolahi and Soolari[320] by determining the amount of tooth staining produced. Tooth staining was reduced by 18% when a dentifrice was used prior to a CHX rinse. However, staining was reduced by 79% when CHX rinsing preceded tooth brushing. It has also been shown that tooth staining by CHX mouth rinses is reduced when rinsing is followed by dentifrice use.[38, 320] Venu and coworkers showed that a dentifrice containing both CHX and SLS reduced *S. mutans* counts for 3 hours while CHX alone had superior anti-bacterial effects and substantivity for 7 hours.[327]

On the other hand, two other previous studies by van Strydonck and coworkers[328, 329] have demonstrated that tooth brushing with a 1.5% SLS-containing dentifrice after a CHX rinse made no difference to the anti-plaque effect of CHX. These studies suggest that while SLS can interfere with the anti-plaque action of CHX, the interference can be obviated by applying CHX prior to the application of SLS. Hence, it is now recommended that there should be an interval period of approximately 30 minutes to 2 hours between brushing with an SLS-containing paste and rinsing with

CHX to ensure the greatest anti-bacterial effect.[320, 323]

10. Conclusions

ECC is a chronic bacterial infection that is still very much present in today's society. The fight against ECC is an uphill battle but targeting children at a young age with preventive strategies will reduce the prevalence of this chronic and destructive disease. As *S. mutans* is one of the main bacteria implicated in ECC, reduction of this bacteria using anti-bacterial agents is a possible method of caries control. The literature supports the use of preventive chemotherapeutics such as fluoride, CHX and PI in order to remove cariogenic bacteria such as *S. mutans* and, hence, reduce the risk of ECC. Combinations of these agents show promise with additional anti-bacerial, anti-plaque and adhesion inhibitory effects. While many anti-bacterial agents have been shown to be effective against *S. mutans*, large scale clinical studies are warranted to determine their safe and efficacious use in children. In addition, further laboratory testing is warranted to examine the possible synergistic or additive effects of combinations of these anti-bacterial agents.

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Chapter 2:

Inhibitory effects of children's toothpastes on *Streptococcus mutans, Streptococcus sanguinis* and *Lactobacillus acidophilus:* A laboratory study

Abstract

Objectives: As suppression of *Streptococcus mutans* in young children may prevent or delay colonisation of the oral cavity, tooth brushing with dentifrices containing anti- *S. mutans* activity may aid in preventing caries. The aims of this study were to compare the effects of children's dentifrices on the growth of *S. mutans* and non-mutans bacteria (*Streptococcus sanguinis* and *Lactobacillus acidophilus*).

Methods: The agar diffusion assay at neutral pH was used to examine the antibacterial activity of commercial dentifrices and their major constituents.

Results: Dentifrices containing 1,450 ppm fluoride produced greater growth inhibition of both *S. mutans* and *S. sanguinis* than those with < 500 ppm. No inhibition was seen for pure solutions of sodium fluoride or sodium monofluorophosphate at fluoride concentrations up to 100,000 ppm. Stannous fluoride exerted antibacterial effects at concentrations above 10,000 ppm. Significant growth inhibition of both *S. mutans* and *S. sanguinis* was seen with sodium lauryl sulphate at 2,500 ppm and with triclosan at 100 ppm. No inhibitory effects were seen for xylitol, sorbitol, sodium pyrophosphate or polyethylene glycol at concentrations up to 80,000 ppm.

Conclusion: Sodium lauryl sulphate is the major bacterial inhibitory compound in children's dentifrices.

Introduction

Worldwide, early childhood caries (ECC) remains difficult to control, with few practical methods available for prevention. As *Streptococcus mutans* is one of the key bacterial species implicated in ECC, its suppression or elimination is a possible method of caries control. As well as providing a physical method for disrupting dental plaque biofilms, dentifrices contain a number of agents which may inhibit the growth of *S. mutans,* including fluoride compounds, surfactants, antiseptics such as triclosan, flavours and humectants. Other dentifrice components such as binders, fillers and abrasives are unlikely to exert antibacterial actions.

Fluoride compounds including sodium fluoride (NaF), sodium monofluorophosphate (NaMFP) and stannous fluoride (SnF_2) have been added to dentifrices for several decades,[1, 2] to help prevent demineralisation of enamel and to enhance remineralisation of early carious lesions. Although fluoride can be bactericidal at high concentrations and particularly under low pH conditions, it is unclear whether any anti-*S. mutans* activity is exerted by fluoride at the levels found in commercial dentifrices used for children.[3, 4] Many "adult" dentifrices contain 1,000 – 1,500 ppm fluoride. In Australia, dentifrices with less than 500 ppm fluoride are recommended for young children due to concerns regarding accidental ingestion and the risk of fluorosis.[5] Dentifrices with fluoride concentrations below 500 ppm have not demonstrated consistent anti-caries effects.[2, 6]

A range of compounds are added to dentifrices to enhance their palatability and cleansing properties, including sugar alcohols such as xylitol as a sweetener (which may also have potential anti-*S. mutans* effects),[7] and sodium lauryl sulphate (SLS) as a surfactant.[8] Other key dentifrice ingredients may include triclosan or stannous fluoride to inhibit plaque formation,[9] and sodium pyrophosphate to reduce calculus formation.[10] We hypothesized that children's dentifrices could exert anti-bacterial activity against *S. mutans* due to the presence of fluoride or other ingredients such as SLS. Although the main properties of toothpastes have been reported by the manufacturers, comparative information among the common toothpastes and details regarding the effects on cariogenic bacteria have not been well researched. Given the clinical importance of tooth

brushing in caries prevention for young children, the aims of this study, therefore, were (i) to investigate the effects of various commercial children's dentifrices on the growth of *S. mutans* and non-mutans bacteria (*S. sanguinis* and *Lactobacillus acidophilus)* and (ii) to determine the antibacterial effects of the various compounds used in dentifrices.

Materials and Methods

Commercially available children's dentifrices with fluoride concentrations of less than 500 ppm were compared to adult dentifrices with 1,450 ppm fluoride and a 5,000 ppm fluoride dentifrice. The details of the products are shown in Table 1.

S. mutans ATCC 25175 and *S. sanguinis* ATCC 10556 were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and maintained in 15% glycerol stocks at -80 °C, while *L. acidophilus* were purchased in capsule form as *L. acidophilus NCFM* (Inner Health Plus, Northgate, Australia). *S. mutans* and *S. sanguinis* were revived from freeze-dried vials and cultured on trypticase soy agar (Becton Dickinson, Franklin Lakes, NJ, USA) supplemented with 5% defibrinated sheep blood (Equicell, Melbourne, VIC, Australia). After the plates had been incubated for 7 days at 37ºC, bacterial colonies were subcultured in brain heart infusion broth (BHI; Becton Dickinson) for 2-3 days at 37 °C. The purity of the plate and broth cultures was monitored by Gram staining and colonial morphology. Bacterial identification was carried out using the API rapid ID 32 Strep and API 50 CHL identification kits (BioMerieux SA, Marcyl'Etoile, France).

Inhibition of growth was assessed for *S. mutans, S. sanguinis* and *L. acidophilus* using the agar diffusion assay, an approach which has been validated in previous investigations.[11-13] In this method, pre-prepared Mueller-Hilton agar petri dishes (MH agar, Thermo Fisher Scientific, Rockford, IL, USA) were employed for *S. mutans* and *S. sanguinis.* This protein-free agar is a standard bacterial medium used for antimicrobial sensitivity testing[14] as it supports the growth of streptococci. For *L. acidophilus,* Rogosa agar (MRS agar, Becton Dickinson, Franklin Lakes, NJ, USA) was used.[15]

In each agar plate, four wells of 5 mm in diameter were formed in the agar by removing plugs cut with a stainless steel punch.[13] The wells were spaced approximately 30 mm apart and 20 mm from the outer edge. All procedures were performed under sterile conditions. Each well was filled with 0.1 mL of dentifrice. For each plate, a mixture of 5 mL aliquots of bacterial suspension ($6x10^8$ bacteria/ mL) and 5 ml of melted Mueller-Hinton agar at 45 °C was poured evenly over the surface of the agar containing wells loaded with the test products.[13]

The culture plates were inverted and incubated at 37 °C for 72 hrs. At the end of this incubation period, bacterial growth was confluent on the agar surface except at areas of growth inhibition, which were indicated by clear areas around the test well containing the relevant product (Fig. 1).[11, 12] The areas of growth inhibition were viewed by inverting the agar plate, and were measured directly using a micrometer gauge. All measurements were performed in triplicate, and the mean and standard deviations calculated. For each agent tested, the experiments were performed in triplicate for *S. mutans, S. sanguinis* and *L. acidophilus*.

Dose response testing was also performed on various compounds found in the dentifrices. NaF, NaMFP and SnF_2 (all from Sigma-Aldrich, St Louis, MO, USA) were used at fluoride concentrations from 100 to 100,000 ppm in distilled water. Sucrose, fructose, xylitol and sorbitol (all from Sigma-Aldrich), were used from 1,000 to 100,000 ppm, and SLS (Amresco Inc, Solon, OH, USA) from 100 to 10,000 ppm. Triclosan (Sigma-Aldrich), sodium pyrophosphate (Alfa Aksar, Heysham, England), and polyethylene glycol 600 (Scharlab S.L., Sentmenat, Spain) were all used from 100 ppm to 8,000 ppm. The test agents were dissolved in 0.15 M phosphate buffered saline, pH 7.2 (PBS), and added into the test wells. Chlorhexidine gluconate (2% w/v) was used as the positive control, and either distilled water or PBS as the negative/vehicle control.

Statistical Analysis

One-way ANOVA with Bonferroni post-tests (for parametric data) and the Kruskal-Wallis test with Dunn post-test (for non-parametric data) were used to test for differences in the zone of inhibition amongst the commercial dentifrices, pure compounds and bacteria.

Statistical analysis was performed using Instat (GraphPad Software Inc., California, USA), and the alpha value was set at 0.05.

Results

Table 2 shows the results for commercial children's dentifrices. Colgate My First and Oral B Stages both inhibited the growth of all three bacteria. Both were significantly better than Macleans Milk Teeth. Greater growth inhibition was seen for *L. acidophilus* than for *S. mutans or S. sanguinis* ($p<0.01$). There were no statistically significant differences in growth inhibition between *S. mutans* and *S. sanguinis.*

Table 3 shows the results for adult dentifrices containing fluoride at 1,450 ppm from NaF, NaMFP or SnF_2, or at 5,000 ppm from NaF. All adult dentifrices gave significantly greater inhibition for *S. mutans* than for *L. acidophilus* ($p<0.01$ for all products). The extent of growth inhibition did not differ between *S. mutans* and *S. sanguinis* for Colgate Total, Oral B Tooth and Gums and Macleans Extreme Clean.

In terms of fluoride levels, dentifrices with less than 500 ppm fluoride gave less growth inhibition against *S. mutans* and *S. sanguinis* than those with 1,450 ppm fluoride ($p<0.01$) (Tables 2, 3 and 4). In contrast, growth inhibition of *L. acidophilus* was similar between < 500 ppm and 1,450 ppm products (Tables 2, 3 and 4). As shown in Table 4, for lactobacilli there were no statistically significant differences between products containing NaMFP (Colgate Pro Relief), NaF (Colgate Total), and SnF_2 (Oral B Pro Health). In contrast, the differences in growth inhibition for *S. mutans* and *S. sanguinis* among these various products were all statistically significant (all $p<0.01$).

Across the study, Colgate Total produced the greatest mean growth inhibition on *S. mutans* and *S. sanguinis,* while Colgate Pro Relief produced the least. For Colgate Total the inhibition zone for *L. acidophilus* was less than half of that seen for *S. mutans* (16.8 ± 1.8 versus 44.6 ± 2.3 mm respectively, $p<0.01$) (Tables 2 and 3).

In terms of the effects of pure compounds, dose response experiments using fluoride solutions at concentrations between 100 and 100,000 ppm failed to show growth inhibition of any three bacterial species for both NaF and NaMFP, while SnF_2 inhibited bacterial growth at a minimum inhibitory concentration (MIC) of 10,000 ppm. With SnF_2, growth inhibition was statistically different between *S. mutans* and *L. acidophilus* at concentrations of 10,000 ppm, 15,000 ppm and 40,000 ppm (Table 5).

The dose response experiments using fructose, sucrose, xylitol, sorbitol and mannitol at concentrations ranging from 100 to 8,000 ppm did not reveal any growth inhibition. The same trend was found for polyethylene glycol and sodium pyrophosphate. In contrast, significant growth inhibition was seen for SLS for all three bacterial species (Table 6). The MIC was 500 ppm for *L. acidophilus,* and 2,500 ppm for both *S. mutans* and *S. sanguinis.* Greater inhibition zones were seen for *L. acidophilus* than for *S. mutans* ($p<0.001$), but differences between the two streptococci were not significant. Triclosan showed an inverse pattern to SLS, in that it inhibited the growth of both *S. mutans* and *S. sanguinis* (with an MIC of 100 ppm), but was ineffective against *L. acidophilus* (Table 7).

Discussion

The results of this study provide several insights into the effects of dentifrices and their components on *S. mutans*. There is increasing evidence that mechanical oral hygiene through tooth brushing is associated with reduced risk for early childhood caries.[16, 17] As most children brush their teeth with a dentifrice, caries preventive effects could arise from both the fluoride and non-fluoride components of the dentifrice. While the major mechanism of caries reduction by fluoride is remineralisation of early lesions and prevention of demineralisation,[18, 19] fluoride can exert antibacterial effects when present at high levels and particularly under acidic pH conditions.[3] The importance of pH is seen in that under the conditions used in this study (neutral pH), both NaF and NaMFP used at levels nearly 10 times greater than those found in dentifrices did not inhibit the growth of *S. mutans, S. sanguinis* or *L. acidophilus.*

Under low pH conditions, fluoride is most able to bind to tooth surfaces and to bacterial cells.[20] Fluoride is able to bind to specific enzymes and proteins to inhibit their actions, and this occurs to a greater extent in acidic environments,[4] due to the formation of hydrogen fluoride,[20] which crosses the bacterial cell membrane readily to dissociate in the cytoplasm. The released fluoride acts as a metabolic inhibitor while the protons acidify the cytoplasm, leading to cell death.[4, 21] Since the current study used neutral pH conditions, the known antibacterial actions of fluoride on *S. mutans* were not seen because the Mueller-Hinton agar has a pH of 7.2-7.4.[22] At this pH, fluoride is less active, and less able to interfere with acid production and proliferation of bacteria.

It is also worth noting that, regarding the antimicrobial effects of fluoride, it is often difficult to apply the in-vitro results to the in-vivo situation.[23] Most investigations have demonstrated that the concentrations of fluoride found in plaque cannot influence the microbial composition.[24] Furthermore, the fluoride concentrations attained in plaque after application of 1500 ppm fluoride toothpaste do not appear to have anti-microbial effects on plaque bacteria.[25]

An interesting point of comparison is that stannous fluoride exerted antibacterial effects at neutral pH, which can be attributed to the action of the stannous ion, which has known antibacterial properties.[26, 27] SnF_2 is a more potent antibacterial agent than NaF, with a lower minimum inhibitory concentration.[28] Clinical studies have shown that suppression of *S. mutans* levels when used in mouth rinse and dentifrice formulations, with a degree of substantivity due to up to 25% of the stannous ion remaining in the oral cavity for up to 4 hours post rinsing.[29] The stannous ion binds to bacterial cell walls and penetrates into bacterial cells.[30] The bound stannous ions may impair bacterial attachment, while internalized ions block functional sites of enzymes and precipitate proteins,[31] thereby interfering with growth and reducing cell viability.[30 32] Together, these actions explain the greater growth inhibition of *S. mutans* produced by SnF_2 than NaF seen in the current study.

An important observation in the present study was the strong effects exerted on the growth of *S. mutans* by SLS. This agent is known to reduce lactate production, glucosyltransferase activity and extracellular polysaccharide formation by *S. mutans.*[8, 33]

It also interferes with the adsorption of proteins to enamel, thus impairing the formation of salivary pellicle.[34] In the present study, SLS gave growth inhibition against all three bacteria tested, with an MIC of 2,500 ppm against *S. mutans* and *S. sanguinis* and 500 ppm against *L. acidophilus.* This anti-mutans activity of SLS may explain past clinical outcomes of low or zero levels of *S. mutans* after the commencement of tooth brushing with children's dentifrice,[16] and fewer mutans streptococci positive children in those young children who had their teeth brushed twice daily with a children's dentifrice compared to those who had less frequent tooth brushing.[35]

Triclosan is well known to exert broad-spectrum antibacterial activity against both Gram negative and Gram positive bacteria including *S. mutans,*[9] by disrupting bacterial cytoplasmic membranes and blockading fatty acid biosynthesis.[36] It interferes with bacterial adhesion and growth.[9] In the present study, triclosan gave an MIC of 100 ppm for *S. mutans* and *S. sanguinis*, but did not inhibit the growth of *L. acidophilus* at levels below 8,000 ppm. Due to its limited water solubility and low retention in the oral cavity,[37] in some commercial dentifrices such as Colgate Total, triclosan is combined with a copolymer of polyvinylmethylether/ maleic acid (PVM/MA), giving enhanced substantivity and anti-plaque efficacy.[36, 38] In the present study, both dentifrices containing triclosan (Colgate Total and Oral B Tooth and Gums*)* produced statistically greater growth inhibition on all three bacteria compared to the pure triclosan solutions tested, which can be explained by the presence of SLS in the dentifrices. These two dentifrices gave the greatest growth inhibition for *S. mutans* and *S. sanguinis* seen amongst the commercial dentifrices tested. These streptococci have similar structural properties,[39, 40] so this is not unexpected.

However, the antimicrobial effects of triclosan are likely to be diluted to sub-lethal concentrations when mixed with saliva in the mouth. As the use of sub-lethal concentrations of triclosan may promote the development of cross or co-resistance to other clinically important antimicrobial agents, some authors have proposed that the use of triclosan be restricted to well documented purposes.[41]

While many studies have reported that regular tooth brushing lowers caries risk, it is unclear to what extent this relates to gross disruption of the dental plaque biofilm,

selective suppression of cariogenic bacteria, or remineralisation from fluoride. The present results indicate that some dentifrice components such as SLS, triclosan and stannous fluoride can exert inhibitory properties on bacterial growth, whilst others such as xylitol and sorbitol (which are added to improve taste), polyethylene glycol (a humectant) and sodium pyrophosphate (an anti-calculus agent) do not, in the assay system used. Past studies have shown that xylitol and sorbitol are metabolised only minimally by the test bacteria used in this study.[7, 42] The agar diffusion assay does not rely upon bacterial adhesion, which is why agents which can block binding to apatite crystals sites, such as sodium pyrophosphate[10] and polyethylene glycol (PEG)[43, 44] did not show antibacterial activity.

While the present laboratory results provide insight into the antibacterial activity of children's toothpaste, the clinical implications were not investigated. In the mouth, saliva is likely to influence the activity of the active components through modulation of the pH or dilution of the compounds to less effective concentrations. Furthermore, the laboratory exposure time of 72 hours may not be reached in the mouth due to clearance of the agent from salivary flow.

In conclusion, inhibiting the growth of *S. mutans* in young children during the early stages of colonisation may prevent its long term establishment in the oral cavity. To this end, tooth brushing with dentifrices containing compounds with anti-*S. mutans* activity can contribute to the prevention of early childhood caries. The present study shows that children's dentifrices can inhibit the growth of *S. mutans*, and this is largely due to sodium lauryl sulphate.

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Tables and Figures

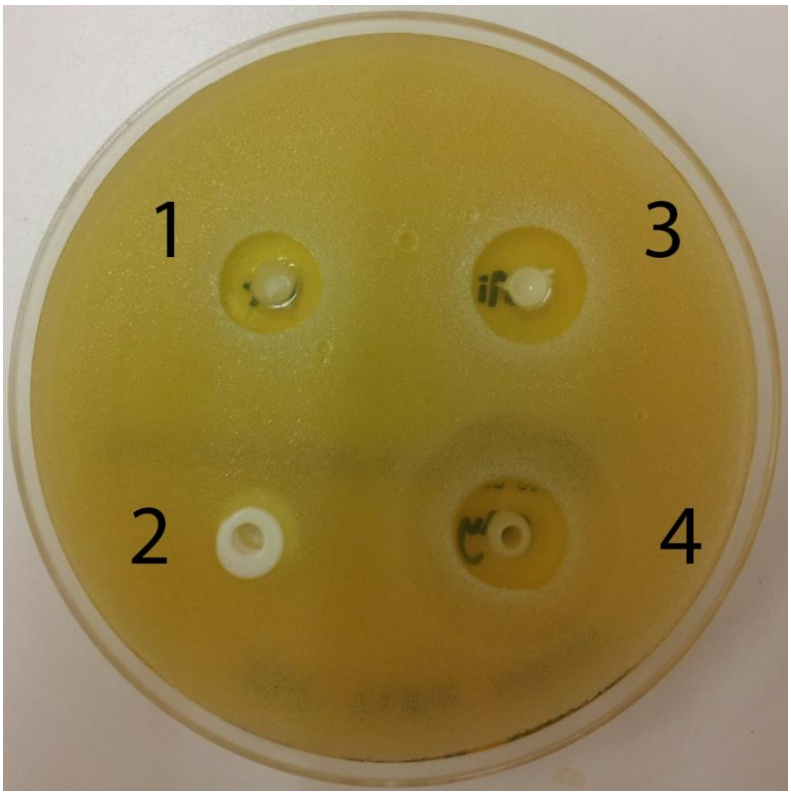

Figure 1. A culture plate with wells overlaid with *S. mutans*. 1 = Oral B Stages, 2 = 0.1% sodium fluoride, 3 = Macleans Milk Teeth, and 4 = Colgate My First. Clear zones of growth inhibition are observed around wells 1, 3 and 4, but none around well 2.

Table 1. Fluoride compounds and other ingredients in dentifrices commercially available in Australia

	Product	Fluoride	Other ingredients (listed by manufacturer)
Standard adult fluoride concentration dentifrice	Colgate Total (Colgate Palmolive Pty Ltd, Sydney, Australia)	Sodium fluoride (1,450 ppm F)	Water, Hydrated silica, Glycerin, Sorbitol, PVM/MA, Copolymer, Sodium lauryl sulfate, Flavour, Carrageenan, Titanium dioxide, Sodium saccharin, Triclosan
	Oral B Tooth and Gums (Procter & Gamble, Cincinnati, USA)	Sodium fluoride (1,450 ppm F)	Aqua, Hydrated silica, Sorbitol, PEG-6, Sodium lauryl sulphate, Tetrapotassium pyrophosphate, Disodium pyrophosphate, Tetrasodium pyrophosphate, Aroma, Cellulose gum, Cl77891, Xanthan gum, Carbomer, Sodium saccharin, Triclosan, Limonene
	Macleans Extreme Clean (GlaxoSmithKline Pty Ltd, Melbourne, Australia)	Sodium fluoride (1,450 ppm F)	Water, Sorbitol, Hydrated silica, Glycerin, PEG-6, Sodium Lauryl Sulphate, flavour, Xanthan Gum, Titanium Dioxide, Cocamidopropyl Betaine, Sodium Saccharin, Sodium Fluoride, C177492, C173360.
	Colgate Sensitive Pro Relief (Colgate Palmolive Pty Ltd, Sydney, Australia)	Sodium Monofluorophosphate (1,450 ppm F)	Calcium carbonate, Water, Sorbitol, Arginine 8%, Bicarbonate, Sodium lauryl sulphate, Flavour, Sodium silicate, Carmeliose sodium, Sodium bicarbonate, Titanium dioxide, Acesulfame potassium, Xanthan gum, Sucralose, Limonene
	Oral B Pro Health (Procter & Gamble, Cincinnati, USA)	Stannous Fluoride (1,100ppm), Sodium fluoride (350 ppm)	Hydrated silica, Sodium phosphate dodecahydrate, Glycerin, Propylene glycol
Low dose fluoride dentifrice	Colgate My First (Colgate Palmolive Pty Ltd, Sydney, Australia)	Sodium Monofluorophosphate (500 ppm F)	Water, Sorbitol, Hydrated silica, PEG-12, Cellulose gum, Sodium lauryl sulphate, Flavour, Sodium saccharin, Tetrasodium pyrophosphate, FD&C blue no.1
	Oral B Stages (Procter & Gamble, Cincinnati, USA)	Sodium fluoride (500 ppm F)	Sorbitol, Aqua, Hydrated silica, Sodium lauryl sulphate, Cellulose gum, Aroma, Sodium saccharin, Carbomer, Limonene, Trisodium phosphate,Cl42090
	Macleans Milk Teeth (GlaxoSmithKline Pty Ltd, Melbourne, Australia)	Sodium fluoride (500 ppm F)	Water, Sorbitol, Hydrated silica, Glycerin, PEG-6, Sodium lauryl sulphate, Titanium dioxide, Xanthan gum, Genuvisco, Sodium saccharin, Flavour
High Fluoride Specialty Toothpaste	Colgate Neutrafluor 5000 (Colgate Palmolive Pty Ltd, Sydney, Australia)	Sodium fluoride (5,000 ppm F)	Aqua, Silicon dioxide, Sorbitol, Macrogol 600, Carrageenan, Sodium lauryl sulphate, Flavour, Poloxamer, Cocamidopropyl, Betaine, Sodium saccharin, Mica, Sodium hydroxide, Titanium dioxide, Brilliant blue FCF, Quinoline Yellow

Table 2. Growth inhibitory effects of children's dentifrices with 500 ppm F

		Zone of inhibition Mean diameter (mm ± S.D.)				P-Value	
Children's Toothpaste	Fluoride compound	*S. mutans* (SM)	*S. sanguinis* (SS)	*L. acidophilus (LB)*	n^t	SM v SS	SM v LB
Colgate My First	NaMFP (500 ppm F)	12.0 (±0.85)	12.1 (±1.15)	14.7 (±1.34)	12	N.S.*	<0.001*
Oral B Stages	NaF (500 ppm F)	11.7 (±0.69)	11.6 (±0.77)	14.1 (±0.76)	12	N.S.*	<0.001*
Macleans Milk Teeth	NaF (500 ppm F)	13.3 (±0.69)	13.0 (±0.54)	17.3 (±2.66)	12	N.S.*	<0.001*
Mean diameter (mm ± S.D.)		12.4 (±1.02)	12.3 (±1.03)	15.4 (±2.24)			
P value (Colgate My First v Oral B Stages)		N.S.*	N.S.^	N.S.^			
P value (Colgate My First v Macleans Milk Teeth)		<0.01*	<0.05^	<0.01^			
P value (Oral B Stages v Macleans Milk Teeth)		<0.001*	<0.001^	<0.001^			

* Kruskal-Wallis test with Dunn post-tests
^ One-way ANOVA with Bonferroni post-tests
n^t = number of experiments
N.S. = non-significant (>0.05)

Table 3. Growth inhibitory effects of adult strength dentifrices with 1450 ppm F

		Zone of inhibition Mean diameter (mm ± S.D.)				P-Value	
Toothpaste	Fluoride compound (concentration)	*S. mutans* (SM)	*S. sanguinis* (SS)	*L. acidophilus (LB)*	n^t	SM v SS	SM v LB
Colgate Total	NaF (1,450 ppm F)	44.6 (±2.33)	43.3 (±2.02)	16.8 (±1.80)	12	N.S.^	<0.001^
Oral B Tooth and Gum	NaF (1,450 ppm F)	33.5 (±2.07)	35.2 (±2.20)	15.8 (±0.96)	12	N.S.*	<0.01*
Macleans Extreme Clean	NaF (1,450 ppm F)	8.4 (±0.93)	8.0 (±1.32)	14.6 (±2.36)	12	N.S.*	<0.001*
Mean (Colgate, Oral B & Macleans 1,450 ppm NaF)		28.8 (±15.45)	28.8 (±15.41)	15.7 (±1.98)			
Neutrafluor 5000	NaF (5,000 ppm F)	9.8 (±0.50)	9.2 (±0.45)	13.0 (±1.31)	12	N.S.*	<0.01*
Colgate Pro Relief	NaMFP (1,450 ppm F)	13.8 (±1.12)	11.6 (±1.51)	17.9 (±0.54)	12	N.S.*	<0.01*
Oral B Pro Health	SnF_2 (1,100 ppm F) NaF (3,50 ppm F)	23.0 (±0.88)	19.3 (±1.48)	19.0 (±0.99)	12	<0.001*	<0.001*

* Kruskal-Wallis test with Dunn post-tests
^ One-way ANOVA with Bonferroni post-tests
n^t = number of experiments
N.S. = non-significant (>0.05)

Table 4. Comparison of inhibitory effects of adult and child dentifrices

	P-Value		
Comparison Groups	*S. mutans*	*S. sanguinis*	*L. acidophilus*
Colgate Total v Oral B Tooth and Gums	<0.05*	<0.05*	N.S.*
Colgate Total v Macleans Extreme Clean	<0.001*	<0.001*	<0.05*
Oral B Tooth and Gums v Macleans Extreme Clean	<0.05*	<0.05*	N.S.*
Colgate Pro Relief v Colgate Total	<0.001*	<0.001^	N.S.*
Colgate Pro Relief v Oral B Pro Health	<0.05*	<0.001^	<0.01*
Oral B Pro Health v Colgate Total	<0.05*	<0.001^	N.S.*
Mean <500 ppm F v Mean 1,450 ppm F	0.014˜	0.014˜	N.S.˜

* Kruskal-Wallis test with Dunn post-tests
^ One-way ANOVA with Bonferroni post-tests
˜ Mann-Whitney Test
N.S. = non-significant (>0.05)

Table 5. Growth inhibitory effects of stannous fluoride

	Zone of inhibition Mean diameter (mm ± S.D.)				P-Value	
Concentration of SnF_2	*S. mutans* (SM)	*S. sanguinis* (SS)	*L. acidophilus* (LB)	n^t	SM v SS	SM v LB
5,000 ppm	0	0	0	12	-	-
10,000 ppm	9.4 (±0.51)	9.4 (±0.67)	8.7 (±0.49)	12	N.S.*	<0.05*
15,000 ppm	11.3 (±0.65)	11.3 (±0.62)	10.6 (±0.51)	12	N.S.*	<0.05*
20,000 ppm	12.6 (±0.51)	12.8 (±0.58)	13.1 (±0.69)	12	N.S.*	N.S.*
40,000 ppm	16.6 (±0.67)	16.8 (±0.58)	17.7 (±0.78)	12	N.S.*	<0.01*
80,000 ppm	21.3 (±0.65)	20.8 (±0.72)	21.1 (±0.90)	12	N.S.*	N.S.*
100,000 ppm	24.1 (±0.67)	23.4 (±1.00)	24.1 (±0.67)	12	N.S.*	N.S*

* Kruskal-Wallis test with Dunn post-tests
n^t = number of experiments
N.S. = non-significant (>0.05)

Table 6. Growth inhibitory effects of sodium lauryl sulphate

	Zone of inhibition Mean diameter (mm ± S.D.)				P-Value	
Concentration of SLS	*S. mutans* (SM)	*S. sanguinis* (SS)	*L. acidophilus* (LB)	n^t	SM v SS	SM v LB
100 ppm	0	0	0	12	-	-
500 ppm	0	0	5.5 (±0.43)	12	N.S.*	<0.001*
1,000 ppm	0	0	7.5 (±0.99)	12	N.S.*	<0.001*
2,000 ppm	0	0	10.0 (±0.72)	12	N.S.*	<0.001*
2,500 ppm	7.0 (±0.85)	7.6 (±0.90)	10.5 (±0.67)	12	N.S.*	<0.001*
3,500 ppm	9.0 (±0.60)	9.7 (0.98)	11.7 (±0.49)	12	N.S.*	<0.001*
5,000 ppm	10.8 (±0.96)	10.1 (±0.76)	13.3 (±0.75)	12	N.S.*	<0.001*
8,000 ppm	12.8 (±0.72)	12.6 (±0.79)	14.7 (±0.75)	12	N.S.*	<0.001*
10,000 ppm	11.2 (±1.53)	12.3 (±0.78)	12.3 (±1.07)	12	N.S.*	N.S.*

* Kruskal-Wallis test with Dunn post-tests
n^t = number of experiments
N.S. = non-significant (>0.05)

Table 7. Growth inhibitory effects of triclosan

	Zone of inhibition Mean diameter (mm ± S.D.)				P-Value	
Concentration of Triclosan	*S. mutans* (SM)	*S. sanguinis* (SS)	*L. acidophilus* (LB)	n^t	SM v SS	SM v LB
80 ppm	0	0	0	12	-	-
100 ppm	21.1 (±1.17)	21.4 (±1.56)	0	12	N.S.*	<0.001*
500 ppm	23.4 (±1.08)	24.6 (±1.78)	0	12	N.S.*	<0.001*
1,000 ppm	25.1 (±4.21)	27.3 (±2.49)	0	12	N.S.^	<0.001 ^
2,000 ppm	25.6 (±1.68)	29.3 (±2.23)	0	12	<0.05*	<0.01*
5,000 ppm	18.3 (±4.21)	18.7 (±5.82)	0	12	N.S.*	<0.001*
8,000 ppm	21.0 (±3.45)	21.4 (±5.20)	0	12	N.S.^	<0.001 ^

* Kruskal-Wallis test with Dunn post-tests
^ One-way ANOVA with Bonferroni post-tests
n^t = number of experiments
N.S. = non-significant (>0.05)

Chapter 3:

Inhibitory effects of antiseptic mouth rinses on *Streptococcus mutans, Streptococcus sanguinis* and *Lactobacillus acidophilus*

Abstract

Objectives: Oral antiseptics are valuable in controlling oral infections caused by cariogenic bacteria.The aim of this study was to investigate the effects of mouth rinses and pure antiseptic compounds on *Streptococcus mutans* and non-mutans bacteria (*Streptococcus sanguinis* and *Lactobacillus acidophilus*).

Methods: The agar diffusion assay was employed to determine bacterial growth inhibition.

Results: Commercial mouth rinses containing chlorhexidine gluconate (0.2%), cetylpyridinium chloride (0.05%) and sodium fluoride (0.05%) produced statistically similar growth inhibition of *S. mutans, S. sanguinis* and *L. acidophilus* (with zones of inhibition ranging from 7.56 ± 0.52 mm to 7.39 ± 0.53 mm, 17.44 ± 0.94 mm to 18.31 ± 0.62 mm and 8.61 ± 1.43 to 8.67 ± 1.43 mm respectively, $p>0.05$). The chlorhexidine mouth wash produced the greatest mean growth inhibition of *S. sanguinis* and *S. mutans* compared to all other mouth rinses tested ($p<0.01$). The minimum concentrations at which inhibition against *S. mutans* could be detected were chlorhexidine gluconate at 0.005% (wt/vol), cetylpyridinium chloride 0.01% (wt/ vol), povidone iodine 10% (wt/ vol) and sodium hypochlorite 0.5% (vol/vol).

Conclusions: Chlorhexidine (0.01%), cetylpyridinium chloride (0.01%) povidone iodine (10%) and sodium hypochlorite (0.5%) are effective at inhibiting the growth of *S. mutans, S. sanguinis* and *L. acidophilus.*

Introduction

Caries in children is initiated by colonisation of the cariogenic bacteria, mutans streptococci, namely *Streptococcus mutans* and *Streptococcus sobrinus.*[1, 2] Children who develop caries have high mutans streptococci counts and are colonised at younger ages compared to caries-free children.[3] *Lactobacillus* is associated with caries progression due its aciduric and acidogenic properties.[4] There is a great need to find preventive agents for caries which are safe and efficacious for children. Numerous antiseptic agents are potentially useful to reduce oral infection with cariogenic bacteria and control caries in children. Although mouth rinses may not be suitable for children younger than 6 years of age, antiseptic agents can be formulated as gels or added to toothpastes to augment the mechanical removal of bacteria from tooth brushing.

Common antiseptics that are employed in oral hygiene products include chlorhexidine, povidone iodine, cetylpyridinium chloride, sodium hypochlorite and essential oils. Chlorhexidine is a potent, hydrophilic bisguanide antiseptic agent.[5] It reduces plaque formation, and selectively inhibits the growth of Gram-positive cariogenic microorganisms including *S. mutans* and some species of *Lactobacillus*.[5] It binds to negatively charged particles including bacterial cell walls, salivary pellicle and mucosa, giving it high substantivity.[6] At low concentrations (<1%), chlorhexidine is bacteriostatic by interfering with cell wall transportation leading to leakage of intracellular components.[7] At high concentrations (>1%), it is bactericidal by causing precipitation of the intracellular cytoplasm. It also impedes on the action of glycosyltransferase thus preventing adhesion of bacteria to the tooth surface.[7] Numerous studies have shown chlorhexidine in both mouth rinse and toothpaste formulations can suppress mutans streptococci counts in children.[6, 8]

Povidone iodine is an antiseptic with many uses in medicine and dentistry such as reducing bacterial contamination prior to, during and after dental surgery.[9] The complex of iodine with polyvinyl pyrrolidone is used to minimise staining and irritation from the iodine as well as increase its water solubility for enhanced antibacterial actions.[9] Numerous studies have reported that povidone iodine reduces mutans streptococci counts in high caries risk children.[10]

Cetylpyridinium chloride is an antiseptic quaternary ammonium compound with a high affinity for Gram-positive bacteria such as mutans streptococci.[11] As with chlorhexidine, cetylpyridinium chloride has a high binding affinity for negatively charged bacterial cell walls. It causes membrane disruption, leakage of cytoplasmic components and inhibition of metabolism and proliferation. In dental biofilms, it prevents cellular aggregation and thus plaque maturation.[11, 12] Cetylpyridinium chloride has an inherently strong ability to adhere to oral surfaces, although it has limited substantivity.[12]

Essential oils contained in mouth rinses such as Listerine® (Johnson & Johnson Pty Ltd, Melbourne Australia) can permeate through dental biofilms, disrupt bacterial cell walls, inhibit enzyme activity, and prevent bacterial aggregation and proliferation.[13] A few studies have shown that essential oils mouth rinses significantly reduce plaque and total oral bacterial counts [14, 15]

Although the effects of common antiseptics used in oral hygiene products have been well investigated for their clinical effects on periodontal pathogens and *S. mutans*, relatively little is known about their effects on non-mutans streptococci and lactobacilli. Therefore, the aim of the present study was to compare the general effects of mouth rinses and pure antiseptic compounds on *S. mutans* and non-mutans bacteria, *Streptococcus sanguinis* and *Lactobacillus acidophilus* using mono-bacterial cultures.

Materials and methods

Commercially available antiseptic mouth rinses were tested for growth inhibitory effects on *S. mutans, S. sanguinis* and *L. acidophilus*. Brands with alcohol-free formulations were selected for testing except for cetylpyridinium chloride mouth rinses as there were no alcohol-free formulations available commercially. The test mouth rinses include Colgate Savacol® (Colgate Palmolive Pty Ltd, Sydney, Australia), Colgate Neutrafluor® 220 (Colgate Palmolive Pty Ltd), Betadine® (Sanofi-aventis, Virginia, Australia), Cepacol® (Bayer Healthcare, Sydney, Australia), Listerine® Zero (Johnson & Johnson

Pty Ltd, Melbourne Australia) and Milton® solution (Procter & Gamble, Cincinnati, USA). The details of the mouth rinses tested are shown in Table 1.

Dose response testing was performed on pure active ingredients obtained from Sigma-Aldrich (St Louis, MO, USA): chlorhexidine, povidone iodine, sodium hypochlorite, cetylpyridinium chloride and individual essential oils. Solutions of each agent were prepared at concentrations of 0.01%, 0.05%, 0.1%, 0.2%, 0.5% and 0.8% in 0.15M phosphate buffered saline, pH 7.2.

Bacteria were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). *S. mutans* ATCC 25175 and *S. sanguinis* ATCC 10556 were revived from freeze-dried vials. *L. acidophilus* was purchased in capsule form as *L. acidophilus NCFM* (Inner Health Plus, Northgate, Australia). The bacteria were cultured on tripticase soy agar (Becton Dickinson) supplemented with 5% defibrinated sheep blood (Equicell, Melbourne, Australia). The plates were incubated for 7 days at 37ºC. The bacterial colonies were subcultured in brain heart infusion broth (BHI; Becton Dickinson) and incubated for 2-3 days at 37 °C. Viability was checked by subculture, and the purity of the plate and broth cultures was monitored by Gram stain and colonial morphology. Bacterial identification was carried out using the API rapid ID 32 Strep and API 50 CHL identification kits (BioMerieux SA, Marcyl'Etoile, France). All strains were maintained in 15% glycerol stocks at -80 °C.

The inhibition of growth of *S. mutans, S. sanguinis* and *L. acidophilus* from each mouth rinse or pure compound was tested using the agar diffusion assay, an approach which has been validated in previous reports. [16-18] In this method, pre-prepared Mueller-Hilton agar petri dishes (MH agar, Thermo Fisher Scientific, Rockford, IL, USA) were employed for testing *S. mutans* and *S. sanguinis.* This protein-free agar is a standard bacterial medium for investigations of antimicrobial sensitivity testing[19] for *Streptococcal* species. For testing of *Lactobacilli* species*,* Rogosa agar (MRS agar, Becton Dickinson, Franklin Lakes, NJ, USA) was used.[20] In each agar plate, four wells of 5 mm in diameter were formed in the agar by removing plugs cut with a stainless steel borer.[16] The wells were spaced approximately 30 mm apart and 20 mm from the outer edge. All procedures were performed under sterile conditions. Each well was loaded with a fine spiral of filter paper

weighing 0.03g. Sixty µL of mouth rinse or pure compound was pipetted into each well containing the filter paper.

Five mL aliquots of a bacterial suspension ($6x10^8$ bacteria/mL) were added to five mL of melted Muller-Hinton agar at 45 °C, then mixed thoroughly and poured evenly over the surface of each agar plate containing wells loaded with test medicaments.[16] The culture plates were inverted and incubated at 37 °C for 72 hrs. At the end of incubation time, bacterial growth was confluent on the agar surface except at areas of growth inhibition where there was a clear zone surrounding the test well (Figure 1).[17, 18] The areas of growth inhibition were viewed by inverting the agar plate, and were measured directly using a micrometer gauge. All experiments and measurements were performed in triplicate, and the mean and standard deviations obtained. Phosphate buffered saline (PBS) was used as a negative control.

Statistical analysis

One-way ANOVA with Bonferroni post-tests (for parametric data) and the Kruskal-Wallis test with Dunn post-tests (for non-parametric data) were used to test for differences in zone of inhibition among the mouth rinses, individual compounds and bacteria. Statistical analysis was performed using GraphPad (GraphPad Software Inc., California, USA), and the alpha value was set at 0.05.

Results

Results from screening various commercial brands of antiseptic mouth rinses are shown in Table 2. Neutrafluor 220, Savacol and Cepacol were the only products to produce growth inhibitory effects against *S. mutans* and *S. sanguinis.* These mouth rinses produced similar growth inhibition of *S. mutans* and *S. sanguinis* ($p>0.05$). Savacol produced the greatest mean growth inhibition on *S. sanguinis* and *S. mutans* (18.2 ± 0.78 mm and 17.2 ± 0.96 mm respectively) by comparison to Neutrafluor 220 (7.5 ± 0.67 mm and 7.5 ± 0.69 mm respectively) and Cepacol (8.7 ±1.42 mm and 8.6 ± 1.33 mm respectively, all $p<0.01$).

L. acidophilus was inhibited by all mouth rinses tested. The greatest zones of inhibition were achieved by Listerine (15.3 ± 1.70 mm) and Savacol (14.4 ± 0.95 mm, p>0.01). The smallest zones of inhibition were produced by Betadine (7.4 ± 0.77 mm), and Milton (7.5 ± 0.69 mm, p>0.01). Neutrafluor 220 and Cepacol showed statistically greater growth inhibition on *L. acidophilus* than *S. mutans* (12.0 ± 0.69 mm v 7.5 ± 0.69 mm, 11.1 ± 0.51 mm v 8.6 ± 1.33 mm respectively, p<0.01). Savacol produced significantly less growth inhibition on *L. acidophilus* than on *S. mutans* (14.4 ± 0.95 mm v 17.2 ± 0.96 mm, p<0.01).

Results from the chlorhexidine dose response experiments are shown in Table 3. chlorhexidine in solution at all concentrations from 0.01% to 0.8% inhibited growth of *S. mutans, S. sanguinis* and *L. acidophilus,* with increasing zones of inhibition observed with increasing concentrations of chlorhexidine. *S. sanguinis* underwent the greatest growth inhibition from 11.2 ± 0.86 mm at 0.01% to 21.8 ± 0.75 mm at 0.8%. *L. acidophilus* showed the least growth inhibition from 7.5 ± 0.50 mm at 0.01% to 16.3 ± 0.45 mm at 0.8%. There was a statistically significant difference in growth inhibition of *S. mutans* and *L. acidophilus* for all concentrations (p<0.05).

Table 4 shows the results of the dose response experiments using sodium hypochlorite. The minimum inhibitory concentration of sodium hypochlorite on *S. mutans, S. sanguinis* and *L. acidophilus* was 0.5% which produced a mean inhibition diameter of 5.5 ± 0.52 mm, 5.7 ± 0.78 mm and 10.8 ± 1.11 mm respectively. The greatest growth inhibitory effect of sodium hypochlorite was on *L. acidophilus* (10.8 ± 1.12 mm at a concentration of 0.5% to 20.8 ± 0.72 mm at a concentration of 4%). There was no difference in growth inhibition between *S. mutans* and *S. sanguinis* except at a concentration of 5% where there was significantly greater inhibition of *S. mutans* (18.2 ± 0.72 mm v 14.3 ± 0.78 mm, p<0.01).

Table 5 shows the results of the dose response experiments using povidone iodine. The minimum inhibitory concentration for povidone iodine on *S. mutans, S. sanguinis* and *L. acidophilus* was 10%, which produced inhibition of 8.1 ± 1.17 mm, 8.1 ± 0.67 mm and 12.1 ± 2.15 mm respectively. Growth inhibition continued to increase with increasing concentration of povidone iodine for all bacteria. There were no statistically significant

differences in growth inhibition on *S. mutans* and *S. sanguinis* at concentrations of 10%, 20% and 40% ($p>0.05$). At the high concentration of 80%, there was greater inhibition on *S. mutans* compared to *S. sanguinis or L. acidophilus* (20.4 ± 1.68 mm for *S. mutans*, 18.7 ± 1.07 mm for *S. sanguinis*, 17.4 ± 1.83 mm for *L. acidophilus*, $p<0.05$).

The results of the dose response experiments using cetylpyridinium chloride are shown in Table 6. The minimum inhibitory concentration for cetylpyridinium chloride on *S. mutans, S. sanguinis* and *L. acidophilus* was 0.01%, which gave inhibition zones of 6.0 ± 0.00 mm, 5.9 ± 0.23 mm and 7.9 ± 1.00 mm respectively. Growth inhibition increased with higher concentrations of cetylpyridinium chloride for all three bacteria. As shown in Table 6, the growth inhibitory effect on *L. acidophilus* appeared to plateau from a concentration of 0.8% to 1.6% (13.9 ± 0.71 mm to 14.3 ±1.44 mm). There was a statistically significant difference in growth inhibition between *S. mutans* and *S. sanguinis* only at the concentration of 1.6% (16.1 ± 0.90 mm, 13.4 ± 1.44 mm respectively, $p<0.001$). There was a statistically significant difference between *S. mutans* and *L. acidophilus* at all concentrations except for 1.2% ($p>0.05$, Table 6).

The four key active ingredients from essential oils rinses investigated were menthol, thymol, methyl salicylate and eucalyptol. These were tested at a concentration in buffer of 0.1%, as their concentrations were all below 0.1% in Listerine Zero mouth rinse. None of the individual essential oils inhibited the growth of *S. mutans, S. sanguinis* or *L. acidophilus* (Results not shown).

Discussion

As indicated in recent reviews [21-23] and an authoritative textbook on oral microbiology,[24] the antimicrobial effects of oral antiseptics have been well investigated with respect to the periodontal effects in adult populations. By contrast, there is comparatively little information on the use of antiseptics for caries prevention. As *S. mutans* is an important cariogenic bacteria and reducing its counts is associated with a decrease in caries risk,[1, 6] it is worthwhile examining the comparative effects of commercial antiseptics on *S. mutans* and non-mutans bacteria to determine their potential as anti-caries agents.

Of the antiseptics tested in the present study, chlorhexidine showed the greatest activity against both *S. mutans* and *S. sanguinis*. In the experiments using pure chlorhexidine, the extremely low concentration of 0.005% gave approximately the same amount of inhibition as 20% povidone iodine, 0.8% cetylpyridinium chloride and 2.0% sodium hypochlorite. There were no effects on *L. acidophilus* at this low concentration of chlorhexidine, although inhibitory effects were observed at doses of 0.01% and higher. In contrast, the other antiseptics tested produced greater inhibitory effects on *L. acidophilus* compared to that observed with *S. mutans*. The present results are thus supported by our previous clinical studies which reported that the percentage of 3-4 year-old children who eliminated mutans streptococci from their mouths increased from 28% after three months to 48% after six months and over 70% after 12 months of 0.2% chlorhexidine gel use.[6] They also substantiate the results of Emilson and co-workers[25] which showed that after 14 days of applying a 1% chlorhexidine gel, all participants in the study eliminated *S. mutans* from their mouths.

The inclusion of *S. sanguinis* and *L. acidophilus* as test organisms in this study helps to demonstrate the comparative effectiveness of the antiseptics on other oral bacteria besides *S. mutans*. The results showed that at all concentrations, *S. sanguinis* was as susceptible as *S. mutans* to the inhibitory effects of chlorhexidine, and that *L. acidophilus* was also inhibited, although to a lesser extent compared to *S. mutans*. These observations thus suggest that there is a potential for the relative proportions of plaque to change after treatment with chlorhexidine. Our findings regarding *S. sanguinis* are different to those reported in early clinical studies, where *S. sanguinis* increased in proportion in plaque after treatment with chlorhexidine, whilst *Lactobacillus* levels were not affected.[25, 26] These differences found between various experimental methods are most likely explained by the fact that the inhibitory effects on planktonic cells of *S. sanguinis* and *Lactobacilli* used in the present method are negated in the environment of the biofilm mass whereas those of *S. mutans* are not.

By comparison, there is minimal evidence for the use of cetylpyridinium chloride in children as an anti-caries agent, although studies have demonstrated that pre-surgical 0.05% cetylpyridinium chloride mouth rinses by children aged 10-15 years are efficacious

at reducing both aerobic and anaerobic microorganisms.[27] In the present study, the bacterial growth inhibition shown by 0.05% pure cetylpyridinium chloride solution was slightly less than that produced by the commercial product Cepacol containing 0.05% cetylpyridinium chloride, suggesting that the commercial product has additional inhibitory actions over the pure compound, possibly related to the ethanol content, or other components such as surfactants. Our results also demonstrated that pure solutions of cetylpyridinium chloride are effective at concentrations as low as 0.01% suggesting that cetylpyridinium chloride has potential for use in children, although its effects on *S. mutans* are comparatively less than those of chlorhexidine.

Our results are thus supported by clinical studies which showed that a fluoride mouth rinse (Neutrafluor 220) has growth inhibiting effects against *S. mutans*, *S. sanguinis* and *L. acidophilus*.[28-30] In the present study, it is most likely that other antibacterial compounds in the mouth rinse formulation enhanced the action of sodium fluoride. As Neutrafluor 220 contains sodium benzoate, a common food preservative with known bacteriostatic properties,[31] it is highly likely that it contributes to the antibacterial effects observed in this study. These effects will be investigated in follow-up studies where other components of the antiseptics will be evaluated.

Povidone iodine is available in a mouth rinse formulation and as a topical chair-side treatment for prevention of caries. Our present results are supported by clinical studies which show that povidone iodine at concentrations of 10% can inhibit the growth of *S. mutans.* These studies demonstrated that topical applications of 10% povidone iodine is effective at preventing white spot lesions in toddlers when applied every 2 months, even if the child is bottle feeding on cariogenic substrates at naptime.[32, 33] Similarly, a once-only topical application of 10% povidone iodine followed by acidulated phosphate fluoride in children can result in a decrease in mutans streptococci and lactobacilli levels over 3 months, and a 3-monthly topical application of 10% povidone iodine following dental restorative treatment can reduce *S. mutans* levels and prevent further occurrence of caries. [10, 34]

Tanzer et al., achieved bactericidal effects on *S. mutans* using inorganic iodine at concentrations as low as 0.04% and showed the selective suppression of *S. mutans* over

S. sanguinis at these concentrations.[35] In the present study, the compound povidone iodine (iodine with polyvinyl pyrrolidone) was applied in a form that is commonly used in medicine and dentistry. This formulation has fewer of the problematic side effects compared to pure iodine (staining and mucosal irritation), while still being effective at altering biofilm formation.[9] Using this form of povidone iodine, our results show comparable inhibition of *S. mutans* and *S. sanguinis*, and thus contrast with the report of Tanzer et al., who used an inorganic iodine preparation.[35]

Mouth rinses containing essential oils are available commercially, and one of the leading brands is Listerine (Johnson and Johnson, Melbourne, Australia). This commercial product was tested and produced the greatest inhibition on *L. acidophilus* of all mouth rinses tested. However, the principal active agents (menthol, thymol, methyl salicylate and eucalyptol) in pure forms and at higher concentrations compared to those found in Listerine, did not show inhibition of the growth of *S. mutans*, *S. sanguinis* or *L. acidophilus*. There was no surfactant used when preparing these individual agents in the present experiments, so the bioavailability of these agents may have been limited. We did not use a surfactant to dissolve the active agents as it would have itself exerted antibacterial effects.[36]

The bactericidal properties of essential oils have been attributed to their ability to effectively permeate through established plaque leading to inhibition of enzymes.[13] Several studies have shown beneficial effects of essential oils mouth rinses such as significantly reducing total plaque bacterial counts,[14] reducing mutans streptococci levels in teenagers,[37] and improving plaque and bleeding scores in orthodontic patients[38] In our assays, Listerine Zero (no ethanol) mouth rinse was ineffective at inhibiting bacterial growth. However, we did not test the ability of the rinse or its components to permeate plaque, which is a key bactericidal property of essential oils. Our present results suggest that essential oils do not have a dominant role for preventing caries in high risk children.

Sodium hypochlorite was included in the present study as it is commonly used as a disinfectant for baby bottles and pacifiers and may have potential as a disinfectant to prevent the colonisation of cariogenic bacteria in children.[39] Although it has poor palatability as a mouth rinse, its use for treating adult periodontal disease is currently

being reviewed as studies have demonstrated an improvement of plaque and bleeding scores at concentrations as low as 0.05%.[40, 41] The present results showed that 2% sodium hypochlorite in the form of commercial Milton's solution was less effective against *S. mutans*, *S. sanguinis* and *L. acidophilus* compared to the pure sodium hypochlorite at the same concentration. These findings could be due to antagonistic effects of other agents that are present within the commercial formulation such as the metal salts which were not investigated in this study.

Although this study is limited by the testing of planktonic cells rather than a biofilm, the results are valuable for providing an understanding of the basic mode of action of these various products on single bacterial cultures. The agar diffusion method employed in this report measures only growth inhibition of the bacteria, and does not indicate bacteriostatic or bactericidal activity. It is also possible that the test compounds could possess other mechanisms of antibacterial activity such as interference with adherence to the tooth surface or suppression of other bacteria which are not evaluated in the present study. To determine changes in the biofilm after extended use of chlorhexidine and other preventive compounds in children, we are conducting longitudinal clinical and microbiological studies. From the clinical oral microbiological samples obtained in these studies, it may be possible to evaluate the effects of the antiseptics on the biofilms in children and determine the persistent microbial populations that are resistant to the effects of the antiseptics.

In conclusion, the present study shows that common antiseptics at low concentrations have the potential to inhibit the growth of cariogenic bacteria. Of the agents tested, chlorhexidine produced the greatest growth inhibition against *S. mutans*, *S. sanguinis* and *L. acidophilus* at the lowest concentration. Further testing of these compounds at low concentrations in gel or paste formulations is required, both in vitro and in clinical settings, to find antibacterial agents suitable for use in children younger than 6 years of age who are unable to use a mouth rinse.

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Tables and Figures

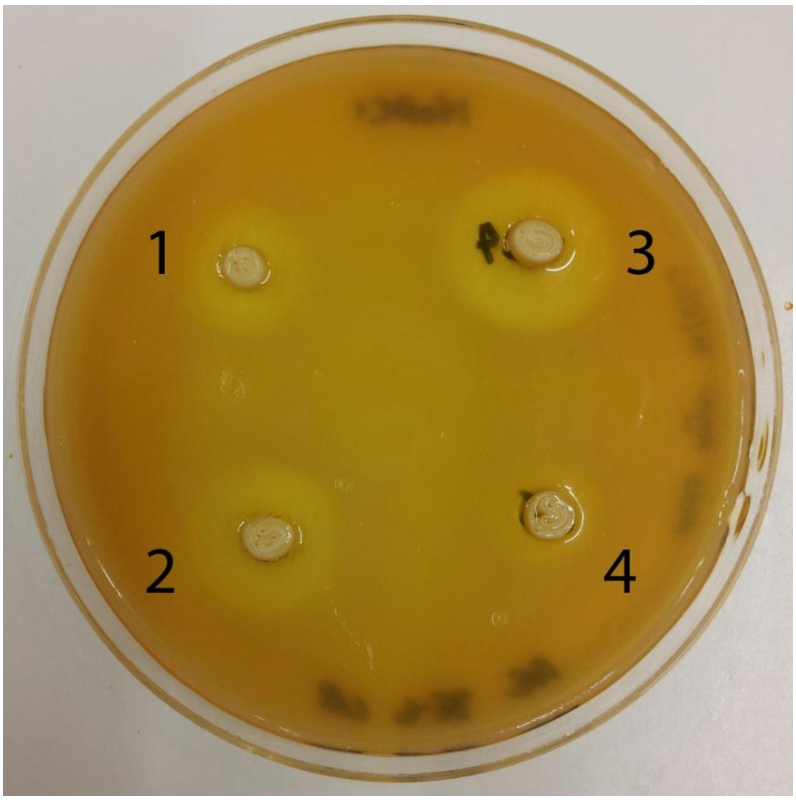

Figure 1. Photograph of a microbial culture plate with wells containing sodium hypochlorite at various concentrations. Well No. 1 contained a pure solution of 1% sodium hypochlorite, Well No. 2 a pure solution of 4% sodium hypochlorite, Well No. 3 a pure solution of 2% sodium hypochlorite, and Well No. 4 contained a pure solution of 0.5% sodium hypochlorite. Clear zones of growth inhibition were observed in all wells. The test bacteria used on this plate was *L. acidophilus.*

Table 1. Active ingredients found in antiseptic mouth rinses commercially available in Australia

Mouth rinse Brand name	Manufacturer (Town, Country)	Active Ingredients	Concentration
Colgate Savacol	Colgate Palmolive Pty Ltd (Sydney, Australia)	Chlorhexidine	2 mg/ml (0.2%)
Colgate Neutrafluor 220 Alcohol Free	Colgate Palmolive Pty Ltd (Sydney, Australia)	Sodium fluoride	0.5 mg/ml (0.05%)
Betadine	Sanofi-Aventis (Virginia, Australia)	Povidone iodine	10 mg/ml (0.1%)
Cepacol	Bayer Healthcare (Sydney, Australia)	Cetylpyridinium chloride	0.5 mg/ml (0.05%)
Listerine Zero	Johnson & Johnson Pacific Pty Ltd (Melbourne, Australia)	Essential oils - menthol (0.042%), thymol (0.064%), methyl salicylate (0.06%), eucalyptol (0.092%)	2.5 mg/ml (0.25%)
Milton	Procter & Gamble (Cincinnati, Ohio, USA)	Sodium hypochlorite	20 mg/ml (2%)

Table 2. Comparison of the growth inhibition by commercial brands of antiseptic mouth rinses on *Streptococcus mutans*, *Streptococcus sanguinis* and *Lactobacillus acidophilus*

		Zone of inhibition Mean diameter (mm ± S.D.)				p-value	
Mouth rinse	Active compound (concentration %)	*S. mutans* (SM)	*S. sanguinis* (SS)	*L. acidophilus* (LB)	n	SM v SS	SM v LB
Colgate Neutrafluor 220	Sodium Fluoride (0.05%)	7.5 (±0.69)	7.5 (±0.67)	12.0 (±0.69)	12	N.S.*	<0.001*
Colgate Savacol	Chlorhexidine (0.2%)	17.2 (±0.96)	18.2 (±0.78)	14.4 (±0.95)	12	N.S.*	<0.01*
Betadine	Povidone Iodine (1%)	0	0	7.4 (±0.77)	12	N.S.*	<0.001*
Cepacol	Cetylpyridinium Chloride (0.05%)	8.6 (±1.33)	8.7 (±1.42)	11.1 (±0.51)	12	N.S.*	<0.001*
Listerine Zero	Essential Oils (0.25%)	0	0	15.3 (±1.70)	12	N.S.*	<0.001*
Milton	Sodium Hypochlorite (2%)	0	0	7.5 (±0.69)	12	N.S.*	<0.001*
	Distilled Water	0	0	0	12	-	-

* Kruskal-Wallis test with Dunn post-tests
N.S. = non-significant (>0.05)

Table 3. Growth inhibition by chlorhexidine on *Streptococcus mutans*, *Streptococcus sanguinis* and *Lactobacillus acidophilus*

	Zone of inhibition Mean diameter (mm ± S.D.)				p-value	
Chlorhexidine (%wt/vol)	*S. mutans* (SM)	*S. sanguinis* (SS)	*L. acidophilus* (LB)	n	SM v SS	SM v LB
0.005%	10.7 (1.15)	9.8 (0.94)	0	12	N.S.*	-
0.01%	10.0 (±0.95)	11.2 (±0.86)	7.5 (±0.50)	12	N.S.*	<0.001*
0.05%	13.3 (±1.27)	16.1 (±0.51)	9.6 (±0.64)	12	<0.05*	<0.05*
0.1%	15.8(±0.62)	17.2 (±0.45)	11.9 (±0.56)	12	<0.05*	<0.05*
0.2%	19.3 (±0.69)	19.5 (±0.45)	13.5 (±0.50)	12	N.S.*	<0.001*
0.5%	19.9 (±0.57)	19.9 (±0.76)	14.5 (±0.72)	12	N.S.*	<0.001*
0.8%	21.1 (±0.64)	21.8 (±0.75)	16.3 (±0.45)	12	N.S.*	<0.001*

* Kruskal-Wallis test with Dunn post-tests
N.S. = non-significant (>0.05)

Table 4. Growth inhibition by sodium hypochlorite on *Streptococcus mutans*, *Streptococcus sanguinis* and *Lactobacillus acidophilus*

	Zone of inhibition Mean diameter (mm ± S.D.)				p-value	
Sodium Hypochlorite (%vol/vol)	*S. mutans* (SM)	*S. sanguinis* (SS)	*L. acidophilus* (LB)	n	SM v SS	SM v LB
0.5%	5.5 (±0.52)	5.7 (±0.78)	10.8 (±1.12)	12	N.S.*	<0.001*
1%	7.3 (±1.42)	7.8 (±1.22)	14.8 (±0.62)	12	N.S.*	<0.001*
2%	10.8 (±0.72)	10.7 (±0.49)	18.2 (±1.19)	12	N.S.*	<0.001*
4%	13.2 (±0.72)	13.5 (±1.51)	20.8 (±0.72)	12	N.S.*	<0.001*
5%	18.2 (±0.72)	14.3 (±0.78)	19.7 (±1.61)	12	<0.01*	N.S.*

* Kruskal-Wallis test with Dunn post-tests
N.S. = non-significant (>0.05)

Table 5. Growth inhibition by povidone iodine on *Streptococcus mutans*, *Streptococcus sanguinis* and *Lactobacillus acidophilus*

	Zone of inhibition Mean diameter (mm ± S.D.)				p-value	
Povidone Iodine (%wt/vol)	*S. mutans* (SM)	*S. sanguinis* (SS)	*L. acidophilus* (LB)	n	SM v SS	SM v LB
<1%	0	0	0	12	-	-
5%	0	0	0	12	-	-
10%	8.1 (±1.17)	8.1 (±0.67)	12.1 (±2.15)	12	N.S.*	<0.001*
20%	9.3 (±0.65)	7.7 (±1.88)	9.6 (±1.00)	12	N.S.*	N.S.*
40%	15.5 (±1.24)	13.9 (±1.31)	12.7 (±1.07)	12	N.S.*	<0.001*
60%	18.7 (±1.61)	15.8 (±0.87)	14.7 (±2.31)	12	<0.01*	<0.001*
80%	20.4 (±1.68)	18.7 (±1.07)	17.4 (±1.83)	12	<0.05^	<0.001^

* Kruskal-Wallis test with Dunn post-tests
^ One-way ANOVA with Bonferroni post-tests
N.S. = non-significant (>0.05)

Table 6. Growth inhibition by cetyl pyridinium chloride on *Streptococcus mutans, Streptococcus sanguinis* and *Lactobacillus acidophilus*

	Zone of inhibition Mean diameter (mm ± S.D.)				p-value	
Cetylpyridinium chloride (%wt/vol)	*S. mutans* (SM)	*S. sanguinis* (SS)	*L. acidophilus* (LB)	N	SM v SS	SM v LB
0.005%	0	0	0	12	NA	NA
0.01%	6.0 (±0.00)	5.9 (±0.23)	7.9 (±1.00)	12	N.S.*	<0.001*
0.05%	7.1 (±0.74)	7.9 (±0.98)	10.9(±0.97)	12	N.S.*	<0.001*
0.1%	7.7 (0.69)	8.4 (±0.53)	11.8 (±1.05)	12	N.S.*	<0.001*
0.2%	8.5 (±0.72)	9.4 (±0.48)	12.5 (±0.69)	12	N.S.*	<0.001*
0.5%	9.6 (±0.73)	9.4 (±0.90)	13.1 (±0.77)	12	N.S.^	<0.001^
0.8%	10.7 (±1.47)	9.8 (±0.69)	13.9 (±0.71)	12	N.S.^	<0.001^
1.2%	12.7 (±0.78)	12.3 (±0.45)	12.6 (±0.51)	12	N.S.*	N.S.*
1.6%	16.1 (±0.90)	13.4 (±1.44)	14.3 (±1.44)	12	<0.001*	<0.05*

*Kruskal-Wallis test with Dunn post-tests
^ One-way ANOVA with Bonferroni post-tests
N.S. = non-significant (>0.05)

Chapter Four:

Interference of antimicrobial activity in combinations of oral antiseptics against *Streptococcus mutans*, *Streptococcus sanguinis* and *Lactobacillus acidophilus*

Abstract

Objectives: The aims of this study were to compare the effects of combinations of sodium fluoride and antiseptic compounds on the growth of *Streptococcus mutans*, *Streptococcus sanguinis* and *Lactobacillus acidophilus*.

Methods: The agar diffusion assay was used to determine bacterial growth inhibition.

Results: Of the combinations tested, 0.1% sodium fluoride with 5% povidone iodine produced synergistic anti-bacterial effects against *S. mutans* and *S. sanguinis.* The combination of 10% povidone iodine with 0.5% sodium hypochlorite produced additive anti-bacterial effects against *L. acidophilus*. Interference was seen in some combinations such as chlorhexidine with sodium lauryl sulphate, most likely through anion-cation reactions. However, 0.1% sodium fluoride when combined with 0.01% chlorhexidine did not interfere with the anti-bacterial effects of chlorhexidine alone against *S. mutans* or *S. sanguinis*, but it reduced the anti-bacterial effects of cetyl pyridinium chloride alone against these bacteria.

Conclusions: Combinations of common antiseptics and fluoride compounds can produce interference, synergistic or additive effects. Of the combinations tested, 0.1% sodium fluoride with 5% povidone iodine has the greatest potential for suppression of *S. mutans*.

Introduction

As *Streptococcus mutans* is one of the key bacterial species implicated in the initiation of early childhood caries, suppressing the levels of this bacterium in children's mouths using antiseptic agents is a possible method of caries control.[1] Although both chlorhexidine (CHX)[2, 3] and povidone iodine (PI)[4-6] have shown potential for reducing early childhood caries, there is a paucity of information on the efficacy of other agents and combinations of various compounds. [2-4] The range of potential agents currently found in mouthwashes and dentifrices includes cetyl pyridinium chloride (CPC),[7-9] sodium lauryl sulphate (SLS)[10] and fluoride compounds, e.g. sodium fluoride (NaF).[11, 12] In addition, sodium hypochlorite (NaOCl) mouth rinses at low concentrations of 0.05% to 0.25% that are harmless to soft tissues, are effective in disrupting dental plaques.[13, 14] Although many of these compounds would be unsuitable for use at high concentrations in mouthwash formulations for children younger than 6 years old, they could be employed in other delivery systems including gels and varnishes, which offer better dosage control.

As low concentrations of active agents improve the safety and reduce the likelihood of adverse effects in children, it is important to utilize where possible combinations of individual compounds at low concentrations that have synergistic or additive anti-bacterial effects. As synergistic agents produce a combined effect greater than the sum of their separate effects, efficacy can be achieved using low concentrations of individual compounds, and thus enhance safety for products intended to be used in children. [15] Combinations which give additive effects are also beneficial. [6]

Relatively few combinations of anti-bacterial compounds have been tested for their inhibitory effects on the growth of *S. mutans*. CPC is not used in combination with CHX, even though they are both cationic antiseptics with similar modes of action and side effects. [16] The combination of CHX and NaF in both dentifrices and mouth rinses has attracted much interest, despite the challenges faced by ionic interactions between them. [17, 18] The interactions of sodium monofluorophosphate (MFP) with CHX appear to be rather different than those of NaF. MFP may show effects on the growth of *S. mutans* comparable to those of CHX.[19]

Although the combined use of NaF and CPC has not yet been evaluated in clinical trials, this combination is likely to be problematic given inactivation of CPC by NaF similar to the pattern seen with CHX when combined with NaF.[9, 19] Likewise, although applications of separate fluoride or iodine containing varnishes may give additive effects on reducing new carious lesions, [5, 6] little is known regarding the use of NaF and PI combined together into a single product.

Sodium hypochlorite (NaOCl) as Milton's solution (one percent NaOCl) is a common disinfectant with broad spectrum anti-bacterial activity, and has been recommended for sterilising baby bottles, baby comforters and toothbrushes.[20, 21] It is also commonly used in endodontics to irrigate root canals. [22] More recently, mouth rinses containing low concentrations of 0.05% to 0.25% NaOCl have been reported to be effective in disrupting dental plaques and preventing gingivitis, with minimal adverse effects on the mucosa.[13, 14] The use of separate applications of NaOCl and CHX in endodontics is well known [23], but this combination has not yet been examined in terms of effects on the growth of *S. mutans*. Likewise, although the synergistic effects of separate applications of PI and CHX have been used in medicine for surgical site antisepsis,[24] how this combination affects the growth of *S. mutans* has not yet been investigated.

The aims of this study, therefore, were to investigate the effects of various combinations of antiseptics and NaF on the growth of *S. mutans* and non-mutans bacteria (*Streptococcus sanguinis* and *Lactobacillus acidophilus)* to determine if the effects of individual antiseptics or combinations are specific to *S. mutans* or extend to non-mutans bacteria. *S. sanguinis* was selected as it is thought to compete with *S. mutans* in the biofilm.[25, 26] We aimed to identify those antiseptics or combinations that have only anti-*S. mutans* activity, as these would reduce numbers of *S. mutans* without affecting the general microbial composition. In addition, we also aimed to identify combinations of compounds that show synergistic or additive effects at relatively low concentrations, as these would be particularly suitable for use in young children.

Methods

Bacterial stocks were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). *S. mutans* ATCC 25175 and *S. sanguinis* ATCC 10556 were revived from freeze-dried vials. *L. acidophilus* was purchased in capsule form as *L. acidophilus NCFM* (Inner Health Plus, Northgate, Australia). The bacteria were cultured on Trypticase soy agar supplemented with 5% defibrinated sheep blood (Equicell, Melbourne, VIC, Australia). The plates were incubated aerobically for 7 days at 37ºC. The bacterial colonies were then subcultured in brain heart infusion broth (BHI; Becton Dickinson, Franklin Lakes, NJ, USA) and incubated for 2-3 days at 37ºC. Viability was checked by subculture, and the purity of the plate and broth cultures was monitored by Gram stain and colonial morphology. Bacterial identification was carried out using the API rapid ID 32 Strep and API 50 CHL identification kits (BioMerieux SA, Marcy l'Etoile, France). All strains were maintained in 15% glycerol stocks at -80ºC.

The compounds tested for inhibition of bacterial growth included NaF, CHX diacetate, PI, CPC and NaOCl (all from Sigma-Aldrich, St Louis, USA), and SLS (Amresco Inc, Ohio, USA). Various combinations of these pure compounds were combined at their known minimum inhibitory concentrations (MIC) in this assay system, using the MIC values established in a pilot study.[27] The extent of inhibition of growth of *S. mutans, S. sanguinis* and *L. acidophilus* by each agent in solution was tested using the agar diffusion assay, [28, 29] with 150 mmol/L phosphate buffered saline pH 7.2 (PBS) as the diluent. In this method, pre-prepared Mueller-Hilton agar petri dishes (MH agar, Thermo Fisher Scientific, Rockford, IL, USA) were employed for testing *S. mutans and S. sanguinis.* This protein-free agar is a standard bacterial medium used for sensitivity testing, and it supports the growth of many bacterial species, including streptococci.[30] For *Lactobacillus* species, Rogosa agar was used (MRS agar, Becton Dickinson).[31] In each agar plate, four wells of 5 mm diameter were formed in the agar by removing plugs cut with a stainless steel punch.[28] The wells were spaced approximately 30 mm apart and 20 mm from the outer edge. All procedures were performed under sterile conditions. Each well was loaded with a fine spiral of filter paper weighing 0.03g, and 60 µl of solution was pipetted into each well. To inoculate the plates, 5 mL aliquots of bacterial suspensions (6×10^8 bacteria/ mL) were added to 5 mL volumes of melted Mueller-Hinton agar at 45 °C, and the resulting mixture mixed thoroughly and poured evenly over the surface of each agar plate containing the wells loaded with the test medicaments.[28] The culture plates were inverted

and incubated aerobically at 37°C for 72 hrs. At the end of the incubation period, bacterial growth was confluent on the agar surface except at areas of growth inhibition, where clear areas around the test wells could be seen clearly.[29] The areas of growth inhibition were viewed by inverting the agar plate, and the diameters of the zones of inhibition measured directly using a micrometer gauge, with measurements being taken three times. Every experiment contained a positive control (CHX), a negative control (normal saline), the single pure compounds and the combinations. These were placed on the same agar dishes. All experiments were performed in triplicate, and means and standard deviations obtained.

In a previous paper, we tested a range of concentrations of the compounds used in the present experiments to determine the concentrations that will produce measureable responses in the agar diffusion system.[32] In the experiments designed to test synergism in combinations of compounds, concentrations at or below the minimum inhibitory levels of the individual compounds were used. The minimum inhibitory concentrations are approximately 0.01% for CPC, 10% for PI, 0.25% for SLS, <0.01% for CHX and <0.5% for NaOCl. [32] In the experiments designed to test additive or interference effects, concentrations that produced measurable effects were selected.

Statistical Analysis

The Kruskal-Wallis test with Dunn post-tests (for non-parametric data) was used to test for differences in the zones of inhibition among preparations and between bacteria. Statistical analysis was performed using GraphPad (GraphPad Software Inc., California, USA) and the alpha value was set at 0.05.

Results

Almost all combinations of the antiseptic compounds and NaF showed similar anti-bacterial effects on *S. mutans* and *S. sanguinis* (Table 1). The only exception to this pattern was the combination of CHX with CPC, which produced greater anti-bacterial effects on *S. sanguinis* than on *S. mutans* (10.2 ± 0.94 mm and 8.9 ± 1.08 mm respectively, $p<0.001$). However, comparing results for *S. mutans* and *L. acidophilus*

revealed statistically significant differences in growth inhibitory zones for all combinations of antiseptic compounds, except for CHX + CPC, CHX + NaOCl, and CHX + PI. Zones of inhibition were greater for *L. acidophilus* than for *S. mutans* for all combinations except NaF with CHX.

The addition of CPC to CHX (Table 2a) did not interfere with the anti-bacterial activity of the CHX against *S. mutans* or *S. sanguinis* but produced a greater anti-bacterial effect against *L. acidophilus* than that found for CHX alone ($p<0.001$). Compared to CPC alone, CHX + CPC produced greater anti-bacterial effects against *S. mutans* ($p<0.01$) and *S. sanguinis* ($p<0.001$), but not *L. acidophilus.*

The combination of CHX + NaOCl (Table 2b) did not show evidence of interference when used against *S. mutans* or *S. sanguinis* as the zones of inhibition were not significantly different for the pure compounds alone versus the combination. However, CHX + NaOCl produced anti-bacterial effects against *L. acidophilus* that were similar to 0.01% CHX on its own, but less than 0.5% NaOCl, indicating interference ($p<0.001$).

The addition of PI to CHX (Table 2c) did not interfere with the anti-bacterial activity of the CHX against *S. mutans* or *S. sanguinis* but enhanced the anti-bacterial effect against *L. acidophilus* ($p<0.05$) over that of CHX alone. Zones of inhibition for *S. mutans* and *S. sanguinis* were greater than for PI alone ($p<0.01$ and $p<0.001$ respectively) but for *L. acidophilus* less than for PI alone ($p<0.05$).

The combination of CHX + SLS (Table 2d) produced anti-bacterial effects against *S. mutans and S. sanguinis* that were less than CHX alone ($p<0.001$) but comparable to SLS alone, indicating interference. No such interference was seen for *L. acidophilus,* for which CHX + SLS was comparable to SLS alone but better than CHX alone ($p<0.001$).

CPC + NaOCl (Table 3a) produced zones of inhibition against *S. mutans* that were similar to either CPC or NaOCl used alone. The anti-bacterial effects of this combination against *S. sanguinis* were greater than CPC on its own ($p<0.001$) but similar to NaOCl used alone, indicating no interference. This same combination, however, produced anti-

bacterial effects against *L. acidophilus* that were comparable to CPC used alone, but less than seen for NaOCl on its own ($p<0.01$), indicating interference.

The combination of CPC + PI (Table 3b) for *S. mutans* was comparable to either CPC or PI alone. For *S. sanguinis* and *L. acidophilus,* the effects were comparable to CPC alone, but less than for PI alone ($p<0.01$), indicating interference.

The anti-bacterial effects of the combination of CPC + SLS (Table 3c) against all three bacteria were greater than for CPC alone but comparable to the effects of SLS alone, indicating no interference.

The combination of NaOCl + PI (Table 4a) did not inhibit the growth of either *S. mutans* or *S. sanguinis*; however, when used alone each individual compound gave inhibition, indicating interference. On the other hand, for *L. acidophilus* the combination of NaOCl + PI produced an enhanced (additive) anti-bacterial effect which was greater than either of the effects produced by individual components., compared to NaOCl ($p<0.01$) or PI ($p<0.001$) used alone.

The anti-bacterial effects of NaOCl + SLS (Table 4b) on the growth of *S. sanguinis* were comparable to NaOCl and SLS used alone. This combination produced zones of inhibition against *S. mutans* that were comparable to NaOCl alone, but additive to those seen with SLS ($p<0.05$). Addition of SLS interfered with the anti-bacterial effects of NaOCl against *L. acidophilus* ($p<0.01$).

The combination of PI + SLS (Table 5) on *S. mutans* and *L. acidophilus* was comparable to either PI or SLS used alone. The anti-bacterial effects against *S. sanguinis* were comparable to 10% PI used alone, but less than SLS used alone ($p<0.01$), indicating interference.

Solutions of NaF did not inhibit the growth of *S. mutans, S. sanguinis* or *L. acidophilus* in this assay system at the concentration used (0.1%). Neither was there any additional effect for *L. acidophilus* of NaF added to CHX, CPC, or PI (Table 6). Adding NaF to CHX did not interfere with the anti-bacterial effects of CHX against *S. mutans* or *S. sanguinis*,

but caused interference for CPC. PI at 5% and NaF at 0.1% have no measurable effects when used individually on *S. mutans* and *S. sanguinis*, but in combination there was inhibition, suggesting the presence of synergism of NaF with PI.

Overall, synergistic anti-bacterial effects were observed only for NaF + PI against *S. mutans* and *S. sanguinis.* There were additive effects of NaOCl + PI against *L. acidophilus*, and NaF + PI against *S. mutans* and *S. sanguinis.* There were several combinations for which interference was noted (Table 7). The compound most likely to be affected was NaOCl, especially when used against *L. acidophilus.*

Discussion

Oral antiseptics have attracted interest as potential anti-caries agents due to the recognition that *S. mutans* is an important cariogenic bacteria in caries initiation and that achieving a reduction in its levels is likely to lead to a lower caries risk.[1] Clinical studies have provided evidence that children who develop caries are colonized by *S. mutans* at younger ages and have higher *S. mutans* counts compared to caries-free children. [33] Furthermore, timely suppression of *S. mutans* in the mouths of young children can reduce caries risk, as demonstrated in studies using chlorhexidine varnish, [2] chlorhexidine gel, [3] and povidone iodine solutions.[4]

Topical fluoride exposure from fluoride dentifrices is well accepted as one of the mainstays of caries prevention for all patients, while in-office applications of fluoride containing varnishes are recommended for high risk children. [11, 12] In addition to topical fluorides, high risk children are likely to benefit from the additional use of anti-bacterial compounds to suppress the growth of cariogenic bacteria. [34] For reasons of safety, the lowest effective concentrations of agents that can be combined with fluoride compounds should be considered for pediatric use. The advantages of combinations with synergistic or additive effects are greater anti-caries and/or anti-plaque effects compared to the individual compounds, whilst reducing dose and therefore enhancing safety. Such strategies for boosting the efficacy and safety of medications are used commonly in medicine and dentistry. For example, the staining and mucosal irritation of pure iodine is

reduced by combining iodine with polyvinyl pyrrolidone. [35] However, in contrast to synergistic and additive effects which are usually beneficial, some combinations of antiseptics can result in interference and reduced efficacy .[36] The results of the present study show that a range of interference issues can occur (Table 7).

The results of the present study provide additional support to the notion that PI can form part of a caries preventive program. To date, PI has been used in children for professional topical applications for control of early childhood caries. The combined use of topical polyvinyl-pyrrolidone-iodine and fluoride varnish can result in more caries-free first permanent molars in children aged 5 to 7 years compared to fluoride varnish alone.[6] Another study by the same research group concluded that a combination of 10% PI and 5% NaF in a varnish reduced the rate of new carious lesions by 31% over a fluoride varnish alone in children aged 1 to 3 years.[5] Initially in this study, 10% PI was combined with 0.1% NaF which produced comparable results to PI alone for all three bacteria. PI and NaF were then combined at concentrations well below those at which the individual compounds are normally used. This produced additive anti-bacterial effects against *S. mutans* and *S. sanguinis,* in agreement with previous studies. Such low dose combinations could have promise for safe use in small children and infants.

Also of interest, NaOCl in combination with PI produced additive anti-bacterial effects against *L. acidophilus.* This combination has not been tested previously as these agents are not commonly used together and NaOCl is only in the early stages of testing for anti-plaque effects.[13, 14] Despite this additive effect against *L. acidophilus*, caution must be used as there was interference between these agents when tested against *S. mutans.*

Overall, of the numerous combinations tested, few produced useful additive anti-bacterial effects over and above those seen from the individual compounds. In most cases, growth inhibition was comparable to the more effective individual compound within the pair used. For example, NaF and CHX produced anti-*S. mutans* effects that were comparable to pure CHX, but greater than that found for NaF alone. These results agree with previous data where varnish and gel formulations showed a useful reduction of *S. mutans* levels and increased enamel mineral density.[17, 18, 37]

In fact, many combinations tested showed interference and reduced efficacy. The results of the present study show that if chlorhexidine (0.01%) is combined with sodium lauryl sulfate (0.25%) or sodium hypochlorite (0.5%) with povidone iodine (10%), or cetyl pyridinium chloride (0.01%) with sodium fluoride (0.1%), the inhibitory activities against *S. mutans* and *S. sanguinis* of the primary compounds will be reduced. These agents are, therefore, not ideal for use in a single anti-bacterial product.

CPC combined with NaF is one such combination that showed interference against both *S. mutans* and *S. sanguinis.* This is in agreement with the known interactions of this molecule with several studies showing antagonism between fluoride toothpaste and CPC mouth rinse use.[8, 16]

Similarly, it is well known that toothpastes containing SLS can impair the antimicrobial effects and substantivity of CHX due to formation of an insoluble salt following the reaction between CHX (a cation) and SLS (an anion).[19] One clinical investigation has showed that rinsing with a 1.5% SLS slurry prior to using a 0.2% CHX rinse reduced the anti-plaque efficacy of the CHX. [38] The results of the present study are in agreement with these findings as SLS interfered with the anti-bacterial properties of CHX against both *S. mutans* and *S. sanguinis* .

The current results also show differences in the responses of individual bacterial species. *L. acidophilus* was inhibited by all the combinations tested, whereas *S. mutans* and *S. sanguinis* were inhibited by most but not all combinations. The comparable effects observed with *S. mutans* and *S. sanguinis* likely reflect the fact that the two *Streptococcal* species have general similar cell wall structural properties.[39] On the other hand, the combinations of CPC + PI and PI +SLS elicited interference on *S. sanguinis* but not on *S. mutans*, suggesting that some differences exist between these bacteria that can lead to different responses with different combinations of antiseptics.[40, 41] Similarly, cell wall and metabolic differences between the streptococci and lactobacilli can cause differences in responses of the bacteria.[42, 43] Although the exact roles of these bacteria in the caries process are not clear, it is generally thought that *S. mutans* is actively involved in the caries process while lactobacilli are likely to be secondary invaders.[44, 45] *S. sanguinis*, on the other hand is thought to exist in competition with *S. mutans* in the dental biofilm, and children with caries have less *S. sanguinis* and more *S. mutans* while the reverse is true for children without caries.[25, 26]

Based on these concepts, the combinations of antiseptics that selectively inhibit *S. mutans*, but not *S. sanguinis* nor lactobacilli would be desirable for removal of *S. mutans* without affecting other plaque bacteria. The combination CPC+ PI meets this criteria as it interfered with the inhibition of *S. sanguinis* and *L. acidophilus* without affecting the inhibitory effects on *S. mutans* of the individual compounds. In contrast, the combination of PI + SLS, while it interferes with inhibition of *S. sanguinis*, does not alter the inhibition of *L. acidophilus* by the individual compounds.

Although the present study is limited by the testing of single bacterial cultures under neutral pH conditions rather than in complex biofilms under varying pH conditions, the results add to the understanding of the antimicrobial effects of the test compounds individually and in combinations.[36] The present results do not, however, indicate the clinical effectiveness of the agents.[36] This is due to the nature of the plaque biofilm and the protection provided by the extracellular matrix which elevates the minimum inhibitory concentrations required for testing biofilms versus planktonic cells.[36] Furthermore, saliva plays an important role in the dilution and clearance of compounds from the oral cavity, and it may interact with antiseptic agents.[36] Hence, clinical trials are necessary to elucidate further the interactions between the antiseptic compounds, saliva and the bacterial biofilm. A further and final point is that the agar diffusion method employed in this study measured only the growth inhibition of the bacteria under neutral pH conditions. It is possible that the individual compounds and combinations tested could possess other mechanisms of anti-bacterial activity at neutral or at other pH values such as interference with adherence to the tooth surface or to other bacteria which could not be investigated in the present assay system.

In conclusion, the present study shows that:

- Combinations of certain common antiseptics and sodium fluoride solutions at low concentrations have the potential to reduce growth of *S. mutans.*
- If chlorhexidine (0.01%) is combined with sodium lauryl sulfate (0.25%) or sodium hypochlorite (0.5%) with povidone iodine (10%), or cetyl pyridinium chloride (0.01%)

with sodium fluoride (0.1%), the inhibitory activities against *S. mutans* and *S. sanguinis* of the primary compounds will be reduced

- Sodium fluoride (0.1%) combined with povidone iodine (5%) show synergistic inhibitory effects against *S. mutans* and *S. sanguinis*. The combination of povidone iodine (10%) and sodium hypochlorite (0.5%) produces additive effects against *L. acidophilus*.
- Further testing is required to determine if these combinations are suitable for use in young children, and whether they are efficacious in preventing early childhood caries.

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Tables and Figures

Table 1. Growth inhibitory effects of combinations of antiseptic compounds and sodium fluoride (NaF)

Compound (Wt/Vol)	Added compound (Wt/Vol)	Zone of inhibition Mean diameter (mm ± S.D.)			p-values	
		S. mutans (SM)	*S. sanguinis* (SS)	*L. acidophilus* (LB)	SM v SS	SM v LB
CHX (0.01%)	CPC (0.01%)	8.9 (±1.08)	10.2 (±0.94)	9.4 (±1.08)	<0.05	N.S.
	NaOCl (0.5%)	9.8 (±0.62)	9.5 (±1.38)	8.6 (±2.31)	N.S.	N.S.
	PI (10%)	9.4 (±1.24)	9.9 (±0.67)	9.6 (±0.90)	N.S.	N.S.
	SLS (0.25%)	7.3 (±1.06)	8.3 (±1.22)	14.3 (±1.54)	N.S.	<0.001
CPC (0.01%)	NaOCl (0.5%)	7.2 (±1.11)	8.8 (±1.06)	11.1 (±1.98)	N.S.	<0.001
	PI (10%)	6.5 (±0.67)	6.2 (±0.39)	10.1 (±1.08)	N.S.	<0.001
	SLS (0.25%)	7.6 (±0.67)	7.5 (±0.67)	14.3 (±1.07)	N.S.	<0.001
NaOCl (0.5%)	PI (10%)	0	0	22.8 (±1.90)	N.S.	<0.001
	SLS (0.25%)	8.5 (±1.17)	8.5 (±1.00)	13.6 (±0.90)	N.S.	<0.001
PI (10%)	SLS (0.25%)	6.7 (±0.65)	7.1 (±0.79)	12.7 (±1.56)	N.S.	<0.001
NaF (0.1%)	CHX (0.01%)	8.8 (±0.83)	9.3 (±0.89)	5.7 (±0.65)	N.S.	<0.001
	CPC (0.01%)	0	0	6.8 (±0.72)	N.S.	<0.001
	PI (5%)	5.2 (±0.39)	5.3 (±0.45)	7.1 (±0.68)	N.S.	<0.001

Table 2. Growth inhibitory effects of chlorhexidine (CHX) with cetyl pyridinium chloride (CPC), sodium hypochlorite (NaOCl), povidone iodine (PI) and sodium lauryl sulphate (SLS)

(a) Growth inhibitory effects of CHX with CPC

	Zone of inhibition Mean diameter (mm ± S.D.)			p-values	
	CHX (0.01% Wt/Vol)	CPC (0.01% Wt/Vol)	Combination CHX + CPC	CHX v Combination	CPC v Combination
S. mutans	10.1 (±1.38)	6.3 (±0.45)	8.9 (±1.08)	N.S.	<0.01
S. sanguinis	10.4 (±0.51)	5.9 (±0.29)	10.2 (±0.94)	N.S.	<0.001
L. acidophilus	7.3 (±0.75)	9.5 (±1.68)	9.4 (±1.08)	<0.001	N.S.

(b) Growth inhibitory effects of CHX with NaOCl

	Zone of inhibition Mean diameter (mm ± S.D.)			p-values	
	CHX (0.01% Wt/Vol)	NaOCl (0.5% Wt/Vol)	Combination CHX + NaOCl	CHX v Combination	NaOCl v Combination
S. mutans	10.1 (±1.38)	9.0 (±2.26)	9.8 (±0.62)	N.S.	N.S.
S. sanguinis	10.4 (±0.51)	8.9 (±1.00)	9.5 (±1.38)	N.S.	N.S.
L. acidophilus	7.3 (±0.75)	15.4 (±1.08)	8.6 (±2.31)	N.S.	<0.001

(c) Growth inhibitory effects of CHX with PI

	Zone of inhibition Mean diameter (mm ± S.D.)			p-values	
	CHX (0.01% Wt/Vol)	PI (10% Wt/Vol)	Combination CHX + PI	CHX v Combination	PI v Combination
S. mutans	10.1 (±1.38)	7.2 (±1.34)	9.4 (±1.24)	N.S.	<0.01
S. sanguinis	10.4 (±0.51)	6.9 (±0.67)	9.9 (±0.67)	N.S.	<0.001

L. acidophilus	7.3 (±0.75)	12.6 (±1.38)	9.6 (±0.90)	<0.05	<0.05

(d) Growth inhibitory effects of CHX with SLS

	Zone of inhibition Mean diameter (mm ± S.D.)			p-values	
	CHX (0.01% Wt/Vol)	SLS (0.25% Wt/Vol)	Combination CHX + SLS	CHX v Combination	SLS v Combination
S. mutans	10.1 (±1.38)	7.0 (±0.74)	7.3 (±1.06)	<0.001	N.S.
S. sanguinis	10.4 (±0.51)	8.4 (±0.67)	8.3 (±1.22)	<0.001	N.S.
L. acidophilus	7.3 (±0.75)	13.3 (±1.07)	14.3 (±1.54)	<0.001	N.S.

Table 3. Growth inhibitory effects of cetyl pyridinium chloride (CPC) with sodium hypochlorite (NaOCl), povidone iodine (PI) and sodium lauryl sulphate (SLS)

(a) Growth inhibitory effects of CPC with NaOCl

	Zone of inhibition Mean diameter (mm ± S.D.)			p-values	
	CPC (0.01% Wt/Vol)	NaOCl (0.5% Wt/Vol)	Combination CPC + NaOCl	CPC v Combination	NaOCl v Combination
S. mutans	6.3 (±0.45)	9.0 (±2.26)	7.2 (±1.11)	N.S.	N.S.
S. sanguinis	5.9 (±0.29)	8.9 (±1.00)	8.8 (±1.06)	<0.001	N.S.
L. acidophilus	9.5 (±1.68)	15.4 (±1.08)	11.1 (±1.98)	N.S.	<0.01

(b) Growth inhibitory effects of CPC with PI

	Zone of inhibition Mean diameter (mm ± S.D.)			p-values	
	CPC (0.01% Wt/Vol)	PI (10% Wt/Vol)	Combination CPC + PI	CPC v Combination	PI v Combination
S. mutans	6.3 (±0.45)	7.2 (±1.34)	6.5 (±0.67)	N.S.	N.S.
S. sanguinis	5.9 (±0.29)	6.9 (±0.67)	6.2 (±0.39)	N.S.	<0.01
L. acidophilus	9.5 (±1.68)	12.6 (±1.38)	10.1 (±1.08)	N.S.	<0.01

(c) Growth inhibitory effects of CPC with SLS

	Zone of inhibition Mean diameter (mm ± S.D.)			p-values	
	CPC (0.01% Wt/Vol)	SLS (0.25% Wt/Vol)	Combination CPC + SLS	CPC v Combination	SLS v Combination
S. mutans	6.3 (±0.45)	7.0 (±0.74)	7.6 (±0.67)	<0.001	N.S.
S. sanguinis	5.9 (±0.29)	8.4 (±0.67)	7.5 (±0.67)	<0.01	N.S.
L. acidophilus	9.5 (±1.68)	13.3 (±1.07)	14.3 (±1.07)	<0.001	N.S.

Table 4. Growth inhibitory effects of sodium hypochlorite (NaOCl) with povidone iodine (PI) and sodium lauryl sulphate (SLS)

(a) Growth inhibitory effects of NaOCl with PI

	Zone of inhibition Mean diameter (mm ± S.D.)			p-values	
	NaOCl (0.5% Wt/Vol)	PI (10% Wt/Vol)	Combination NaOCl + PI	NaOCl v Combination	PI v Combination
S. mutans	9.0 (±2.26)	7.2 (±1.34)	0	<0.001	<0.001
S. sanguinis	8.9 (±1.00)	6.9 (±0.67)	0	<0.001	<0.001
L. acidophilus	15.4 (±1.08)	12.6 (±1.38)	22.8 (±1.90)	<0.01	<0.001

(b) Growth inhibitory effects of NaOCl with SLS

	Zone of inhibition Mean diameter (mm ± S.D.)			p-values	
	NaOCl (0.5% Wt/Vol)	SLS (0.25% Wt/Vol)	Combination NaOCl + SLS	NaOCl v Combination	SLS v Combination
S. mutans	9.0 (±2.26)	7.0 (±0.74)	8.5 (±1.17)	N.S.	<0.05
S. sanguinis	8.9 (±1.00)	8.4 (±0.67)	8.5 (±1.00)	N.S.	N.S.
L. acidophilus	15.4 (±1.08)	13.3 (±1.07)	13.6 (±0.90)	<0.01	N.S.

Table 5. Growth inhibitory effects of povidone iodine (PI) with sodium lauryl sulphate (SLS)

	Zone of inhibition Mean diameter (mm ± S.D.)			p-values	
	PI (10% Wt/Vol)	SLS (0.25% Wt/Vol)	Combination PI + SLS	PI v Combination	SLS v Combination
S. mutans	7.2 (±1.34)	7.0 (±0.74)	6.7 (±0.65)	N.S.	N.S.
S. sanguinis	6.9 (±0.67)	8.4 (±0.67)	7.1 (±0.79)	N.S.	<0.01
L. acidophilus	12.6 (±1.38)	13.3 (±1.07)	12.7 (±1.56)	N.S.	N.S.

Table 6. Growth inhibitory effects of sodium fluoride (NaF) with chlorhexidine (CHX), cetyl pyridinium chloride (CPC) and povidone iodine (PI)

	Compound (Wt/Vol)	Zone of inhibition Mean diameter (mm ± S.D.)		p-values (Compound v Combination)
		Compound	Compound + NaF (0.1%)	
S. mutans	CHX (0.01%)	9.3 (±1.06)	8.8 (±0.83)	N.S.
	CPC (0.01%)	5.8 (±0.58)	0	<0.001
	PI (5%)	0	5.2 (±0.39)	<0.001
S. sanguinis	CHX (0.01%)	9.7 (±0.65)	9.3 (±0.89)	N.S.
	CPC (0.01%)	5.7 (±0.49)	0	<0.001
	PI (5%)	0	5.3 (±0.45)	<0.001
L. acidophilus	CHX (0.01%)	5.3 (±0.45)	5.7 (±0.65)	N.S.
	CPC (0.01%)	6.2 (±1.34)	6.8 (±0.72)	N.S.
	PI (5%)	7.5 (±0.80)	7.1 (±0.68)	N.S.

Table 7. Compounds whose inhibitory effects against certain bacteria were reduced if combined with another compound

Compound (Wt/Vol)	Added compound (Wt/Vol)	Bacteria that had interference effects by the combination
CHX (0.01%)	SLS (0.25%)	*S. mutans* *S. sanguinis*
NaOCl (0.5%)	CHX (0.01%)	*L. acidophilus*
	CPC (0.01%)	*L. acidophilus*
	PI (10%)	*S. mutans* *S. sanguinis*
	SLS (0.25%)	*L. acidophilus*
PI (10%)	CPC (0.01%)	*S. sanguinis* *L. acidophilus*
SLS (0.25%)	PI (10%)	*S. sanguinis*
NaF (0.1%)	CPC (0.01%)	*S. mutans* *S. sanguinis*

Chapter Five:
Discussion and Conclusions

As caries is increasing in prevalence and severity among children, there is great need to find effective preventive agents for children. There is increasing recognition that *S. mutans* is one of the most important cariogenic bacteria and of the concept that its reduction is likely to lead to a decrease in caries risk.[1] It has been shown that children who develop caries are colonised by *S. mutans* at younger ages and have higher counts compared to caries-free children.[2] Timely removal of *S. mutans* from the mouths of young children using good oral hygiene and plaque control can reduce the risk for early childhood caries by preventing their colonisation in the mouth.[3, 4]

Tooth brushing has long been accepted as a mechanism to maintain acceptable oral health and is recommended to all children and adults alike in many countries, including Australia.[5] Since the understanding that caries is associated with increased bacterial levels, toothpaste is also used for enhanced anti-bacterial action. A recent study by Pukallus and coworkers[6] has shown that brushing alone can reduce the risk of caries. This is likely due to the physical disturbance of plaque which relies on good motor control of the tooth brush to engage all areas of plaque accumulation. Children are still developing motor skills so it is recommended that children have their teeth cleaned by a parent until their manual dexterity is improved, around 10 years of age. This requires good compliance by child and parent alike. Close monitoring and direct tooth brushing by the parent depends on the parent making time for oral hygiene over their other parental tasks and employment. It also depends on the child's cooperation with the parent at the time of tooth brushing. Additionally, compliance depends on the taste of the product and ease of application. Children's toothpastes are designed with a sweet taste and minimal foaming effect to address this concern. As compliance is often difficult, the use of a dentifrice containing antiseptic or remineralising agents is recommended for added benefit over brushing alone.

Dentifrices are able to produce anti-bacterial effects throughout the oral cavity due to the foaming action of the dentifrice and don't rely solely on the physical disturbance of the plaque. This is especially important in high caries risk children, whether due to poor diet, poor oral hygiene or developmental anomalies. Tooth brushing is taught from a young age so this is considered an easy and well-accepted method of fluoride application. In high caries risk children, tooth brushing alone may indeed reduce plaque and bacterial

levels, but not to a level low enough to prevent caries development, hence the necessity for an anti-caries agent such as fluoride.

Another parameter to account for when prescribing a caries preventive agent is the physical limitations of the child. Children have a lower body mass and reduced awareness and control over their use of such chemicals. These factors result in greater side effects such as toxicity most commonly due to ingestion. In dentistry, the risk of fluorosis is of concern. Children with a high fluoride intake are at risk of fluorosis which can present as faint white lines or mottling in its milder form to brown staining or pitting with breakdown of the enamel in its more severe form.[7] The benefits and risks must be carefully examined on a case to case basis as some high caries risk children gain great health benefits from the use of a topical fluoride agent of higher fluoride concentration. Furthermore, children must be closely supervised when using a high fluoride concentration product such as mouth rinse or toothpaste to reduce the chances of ingestion. This is especially pertinent when prescribing mouth rinses to small children. Mouth rinses have increased risk of ingestion as children are unaware of the adverse side effects from ingestion and are not adept at spitting out a liquid. One suggestion is that children learn the art of rinsing and spitting out with a safe liquid such as water or milk, prior to the use of a mouth rinse. Varnishes also offer additional caries preventive effects. Similar to tooth brushing, child compliance is of the utmost importance to allow application of the varnish, whether at home or in the dental chair. The decision to use a particular product should be made on a case by case basis weighing up the risks and benefits for that child.

Fluoridated toothpaste is the gold standard in caries prevention with in-office varnishes and mouth rinses used for high risk patients.[8] Fluoride has been shown to have greater anti-bacterial action with higher concentration so it can be recommended that children use small quantities of high fluoride concentration toothpaste.[9, 10] In Australia, however, low dose fluoride toothpastes are commercially available as this lowers the risk of fluorosis in children. Although the primary mechanism of caries reduction by fluoride is remineralisation of early lesions and prevention of demineralisation, it is also often thought that caries reduction may additionally result from the anti-bacterial activity of fluoride.[11] It is thus interesting to note from the results of the present study that NaF and

NaMFP in concentrations up to over nearly 10 times that found in toothpastes did not inhibit the growth of *S. mutans, S. sanguinis* or *L. acidophilus.*

Clinically, the bioavailability of fluoride is of great importance.[12] Bioavailability depends on the mode of application, the solubility of the fluoride compound and the adhesion of fluoride to the tooth surface. Sodium fluoride is highly soluble, so the fluoride ion is able to react with minerals readily to form calcium fluoride at the tooth's surface or fluorapatite within the enamel, and hence produce an enhanced anti-caries effect. Below the critical pH of 5.5, hydroxyapatite usually undergoes demineralisation. However, in the presence of fluoride, there is enhanced remineralisation through the formation of fluorapatite and a raised critical pH for demineralisation.[13] Also at low pH and short exposure times, calcium fluoride compounds form on the tooth's surface which act as a fluoride reservoir and prevents demineralisation.[13] This clearly explains fluorides increased interactions in lower pH environments. This intricate interaction between pH and fluoride may explain the findings in this study as it is in these low pH environments that fluoride is most able to bind to tooth surfaces or bacterial cells.[14] For example, an in vitro study has shown that lower concentrations of fluoride (100 ppm versus 300 ppm) result in spontaneous precipitation of calcium fluoride at lower pH (pH 5.0 versus pH 7.2).[15] This is further corroborated by Rolla who showed fluoride release from calcium fluoride to be pH dependent, with greater fluoride release at low pH, when it is most needed.[16] Koch and coworkers compared the cariostatic effect of toothpaste with different fluoride concentrations and pH. [17] They observed that a toothpaste with 250 ppm F as NaF at pH 5.5 reduced caries development to the same extent as a neutral 1000 ppm NaF. Similarly, Arnold and coworkers showed a dentifrice of amine fluoride at pH 4.5 to be more effective at remineralising enamel than a dentifrice of amine fluoride at pH 6.9.[18] This supports the hypothesis that fluoride is more active in low pH environments.

It has also been shown that *S. mutans* is more sensitive to fluoride in low pH environments and that fluoride-resistant strains of *S. mutans* are less able to maintain acid production in the presence of fluoride.[19, 20] For example, the glycolysis of a fluoride-resistant mutant of *S. salivarius* was not affected by fluoride at pH 7.0, but was inhibited at pH 5.8.[21] This is due to fluoride being able to bind specific enzymes and proteins to inhibit their actions to a greater extent in acidic environments.[22] Furthermore, practically

all the free fluoride ions exist as HF.[14] Due to the weak acid nature of HF, in acidic environments fluoride is able to cross the bacterial cell membrane and dissociate in the cytoplasm. F- acts as a metabolic inhibitor while H+ acidifies the cytoplasm, leading to cell death.[22, 23] Interestingly, this predicts that if an organism does not lower the extracellular pH, then fluoride inhibition of glucose fermentation might not occur. These data support the conclusion that fluoride is able to better inhibit glycolysis under cariogenic conditions, ie in acidic environments.

In this study, the agar diffusion assay was used with Meuller-Hinton agar as the medium for growth of *S. mutans.* Meuller-Hinton agar has a neutral pH of 7.2-7.4.[24] At this pH, fluoride is less active, and less able to interfere with acid production and growth and proliferation of these bacteria. This may explain why NaF was ineffective at inhibiting the growth of *S. mutans* in this assay. It could, therefore, be reasonably expected that greater growth inhibition of *S. mutans* by NaF would occur in an acidic environment.

By comparison, SnF_2, found in several adult toothpaste formulations, did inhibit the growth of these bacteria, as expected.[25, 26] This may be attributed to the tin ion. A study by Ferretti and coworkers showed SnF_2 to be more bacteriostatic and bactericidal than NaF with a lower minimum inhibitory concentration and a reduced amount of soluble glucans and hence reduced bacterial binding to enamel.[27] There was also a high level of tin uptake by *S. mutans* treated with SnF_2 suggesting increased inhibitory properties of the compound. This is further corroborated by Attramadal and Svatun who showed SnF_2 to have a strong anti-bacterial effect against *S. mutans* in mouth rinse and dentifrice formulations with up to 25% of the tin ion remaining in saliva for up to 4 hours post rinsing.[28] Tinanoff and Camosci explored the effects of SnF_2 on *S. mutans* using electron microscopy. They found an increase in tin on the bacterial cell walls as well as intracellularly.[29] The tin on the cell walls was assumed to interrupt bacterial attachment to other bacteria and to tooth structure. Metal ions such as tin can affect growth, morphology and biochemical activity of microbes through the blocking of functional sites, inactivation of enzymes and disruption of cellular membrane integrity.[30] Tin specifically, is known to be germicidal due to its ability to precipitate protein.[30] It has been suggested that tin reduces DNA production by interfering with glycolytic enzymes, hence reducing cell viability.[31] Intracellular tin is also metabolically disruptive so can alter cell

morphology leading to unbalanced growth.[29] This is evident by the greater growth inhibition of *S. mutans* produced by SnF_2 than NaF. However, these two agents have been shown to have comparable effects on acid production,[31, 32] a property not tested in this study.

In contrast to fluoride, the compound in toothpaste that was shown to have considerable anti-bacterial activity is the detergent/surfactant, SLS. In previous studies, SLS has been shown to reduce lactate production and extracellular polysaccharide formation by *S. mutans*.[33] SLS also interferes with the adsorption of proteins to enamel, thus affecting the formation of the salivary pellicle.[34] In the present study, SLS produced growth inhibition against all three bacteria tested. There was a minimum inhibitory concentration of 2500 ppm against *S. mutans* and *S. sanguinis* and 500 ppm against *L. acidophilus.* Our results are thus in agreement with previous data which suggest that SLS can inhibit the growth of *S. mutans,* previously suggested to be closely related to the inhibition of enzymic activities of glucosyltransferases which are involved in glucan synthesis and caries initiation.[35]

Law and Seow[3] reported that a significant number of children infected with *S. mutans* did not show the presence of *S. mutans* after the commencement of tooth brushing with children's toothpaste. Further extending these findings are the results of Plonka and coworkers[2] which demonstrated that there were fewer mutans streptococci positive children in the group who had their teeth brushed twice daily with children's toothpaste compared to the group who had less frequent tooth brushing. These findings are the end result of a complex interaction between the anti-mutans activity of SLS, the enhanced action of fluoride in a potentially low pH intraoral environment and the physical disturbance of plaque by tooth brushing.

Another compound found in toothpaste that was tested in this study was triclosan. This biocide is known to exert broad-spectrum anti-bacterial activity against both Gram negative and Gram positive bacteria including *S. mutans.*[36] Its main mode of action involves disruption of bacterial cytoplasmic membranes and blockade of fatty acid biosynthesis.[37] It is most effective on plaque-free surfaces, which suggests that it interferes with bacterial adhesion and growth.[36] In the present study, triclosan showed a

minimum inhibitory concentration of 100 ppm on *S. mutans* and *S. sanguinis*, although it did not inhibit the growth of *L. acidophilus* at concentrations less than 8,000 ppm. Due to its poor water solubility and low retention in the oral cavity,[38] triclosan is combined with a copolymer of polyvinylmethylether/ maleic acid (PVM/MA) for enhanced substantivity and efficacy in toothpastes.[37, 39] In this study, the toothpastes containing triclosan (*Colgate Total* and *Oral B Tooth and Gums)* produced statistically greater growth inhibition on all three bacteria compared to the pure triclosan solutions tested, which is likely due to the presence of SLS. Furthermore, these two toothpastes produced the greatest growth inhibition against *S. mutans* and *S. sanguinis* compared to the other pastes. The inhibition was more pronounced in the two *Streptococcal* species compared to *L. acidophilus*, suggesting triclosan may have enhanced selective activity against *Streptococcal* bacteria. The present findings are substantiated by two review articles which report clinically significant improvements in plaque control and gingivitis following use of a triclosan-containing dentifrice.[37, 39]

In the case of toothpastes containing triclosan and SLS, the growth of *S. mutans* and *S. sanguinis* were inhibited, probably due to the fact that both *Streptococcal* species have similar structural properties.[40, 41] While most studies have reported that a reduced caries risk is associated with regular tooth brushing, whether this is due more to reduced numbers of cariogenic bacteria, disruption of biofilm, or remineralisation from fluoride is unclear. Furthermore, long-term effects such as changes in the relative proportions of microorganisms in the microbial biofilm, resulting from dentifrices, have not been well investigated.

In this study, other compounds found in toothpastes were also assessed for possible inhibitory properties. These included xylitol and sorbitol, which are added to improve the taste of the pastes, polyethylene glycol, which is added as a humectant and sodium pyrophosphate which is added as an anti-tartar agent. The results did not show growth inhibition from xylitol and sorbitol with *S. mutans, S. sanguinis* and *L. acidophilus*, probably due to the fact that xylitol and sorbitol are metabolised minimally by these bacteria.[42, 43]

Although sodium pyrophosphate is added to toothpaste as a tartar control agent, some authors have proposed that sodium pyrophosphate can reduce bacterial adhesion to tooth surfaces resulting from the blocking of active protein sites on apatite crystals.[44] The results from this study, however, suggest there is no direct growth inhibition of *S. mutans, S. sanguinis* and *L. acidophilus* from sodium pyrophosphate. Similarly, there was no growth inhibition noted with polyethylene glycol (PEG), the humectant used in toothpaste. Although PEG has been shown to reduce bacterial adhesion to enamel surfaces through a high binding affinity for hydroxyapatite crystals,[45, 46] interference with bacterial growth is not a known property of PEG.

More recently, the use of oral antiseptics has been re-focussed as a strategy for caries prevention in young children. Recent systematic reviews have reported on the effectiveness of CHX,[47] CPC [48] and EO [49] for reducing plaque and gingivitis in adult populations. In paediatrics, as in adult dentistry, oral antiseptics are applied mainly to reduce infections before and after oral surgical procedures as well as after oral trauma. In addition, oral antiseptics are used to reduce gingivitis and oral infections in high risk children such as those who are immunocompromised.

Thus far, it is more common to prescribe children at high caries risk two separate products, such as a separate fluoride toothpaste and mouth rinse or additional in-office topical application of another agent such as chlorhexidine. This enables control of the concentration and quantity of product applied. Much data supports the use of a fluoride dentifrice and separate CHX or CPC mouth rinse.[50-52]

Of the antiseptics tested in the present study, CHX showed the greatest activity against *S. mutans* and *S. sanguinis*. In the experiments using the pure compounds, at an extremely low concentration of 0.005%, CHX gave approximately the same amount of inhibition as 20% PI, 0.8% CPC and 2.0% NaOCl. There were no effects on *L. acidophilus* at this low concentration of CHX, although inhibitory effects were observed at doses of 0.01% and higher. In contrast, the other antiseptics tested produced greater inhibitory effects on *L. acidophilus* compared to that observed with *S. mutans*. The present results thus extend our teams previous clinical studies which reported that the percentage of 3-4 year-old children who eliminated MS from their mouths increased from

28% after three months to 48% after six months and over 70% after 12 months of a 0.2% CHX gel use.[53] They also substantiate the results of Emilson and co-workers[54] which showed that after 14 days of applying a 1% CHX gel, all 5 participants in the study eliminated *S. mutans* from their mouths.

The inclusion of *S. sanguinis* and *L. acidophilus* in this study helps to demonstrate the comparative effectiveness of the antiseptics on other oral bacteria besides *S. mutans*. The results showed that at all concentrations, *S. sanguinis* was as susceptible as *S. mutans* to the inhibitory effects of CHX, and that *L. acidophilus* was also inhibited, although to a lesser extent compared to *S. mutans*. These observations thus suggest that there is a potential for the relative proportions of plaque to change after treatment with CHX. These findings regarding *S. sanguinis* are thus different to those reported in early clinical studies where *S. sanguinis* increased in proportion in plaque after treatment with CHX, whilst *Lactobacillus* levels were not affected.[50, 54] These differences are most likely explained by the fact that the inhibitory effects on planktonic cells of *S. sanguinis* and *Lactobacilli* used in the present method are negated in the environment of the biofilm mass whereas those of *S. mutans* are not.

For use in paediatric dentistry, CHX has been shown to be highly effective in reducing early childhood caries when applied in the form of a high concentration (40%) varnish delivered 6 monthly by professionals.[55] On the other hand the present study showed that a concentration as low as 0.01% gave clear evidence of bacterial inhibition, suggesting that very low levels applied frequently (e.g. daily) may be equally effective. However, clinical trials conducted by our team showed that daily application of 0.12% CHX gel did not result in lower caries rates compared to controls, probably due to poor compliance of gel use associated with the unacceptable taste of the gel for young children.[56]

By comparison, there is minimal evidence for the use of CPC in children as an anti-caries agent. Work by Pawha and coworkers[57] demonstrated the clinical effectiveness of a commercially available 0.07% CPC mouth rinse at reducing the plaque index and bleeding scores in children and young adults (10-25 years old) undergoing fixed orthodontic appliance treatment. Further, use of a pre-surgical 0.05% CPC mouth rinse by children aged 10-15 years has shown efficacy at reducing both aerobic and anaerobic

microorganisms.[58] Our results are in agreement with these data in that a 0.05% CPC-containing mouth rinse (Cepacol) was effective in inhibiting growth of *S. mutans*, *S. sanguinis* and *L. acidophilus*.

The bacterial growth inhibition shown by 0.05% pure CPC solution was slightly lower than that by the commercial product Cepacol containing 0.05% CPC, suggesting that the commercial product has additional inhibitory action, possibly related to the ethanol content or another component such as the surfactant. A study by Sreenivasan and coworkers[59] has shown CPC-containing mouth rinses with alcohol to have a reduced minimal inhibitory concentration when inhibiting the growth of *S. mutans* and Gram negative periodontal microorganisms compared to a CPC-containing mouth rinse without alcohol. Hence our results using a commercial CPC preparation containing alcohol provides an unfair comparison with the other non-alcohol containing antiseptic mouth rinses. Our results also revealed pure solutions of CPC to be effective at concentrations as low as 0.01%, suggesting that frequent application of CPC at low concentrations has potential for use in children. The use of CPC in gel or varnish form has thus far only been studied by combining CPC with zinc gluconate in a gel[60, 61] or with acrylic resin in a varnish[62] producing anti-calculus and anti-bacterial effects. Our finding that CPC is less effective than CHX at inhibiting bacterial growth, agrees with work by Nelson-Filho and coworkers[63], which showed that a 0.05% CPC mouth rinse is effective at disinfecting toothbrushes used by preschool-aged children at day-care, although not to the extent of CHX.

The NaF mouth rinse (Colgate Neutrafluor 220) was also found to be effective at inhibiting the growth of *S. mutans*, *S. sanguinis* and *L. acidophilus*. It contains NaF at a concentration of 0.05%, which has both strong remineralisation and bactericidal effects.[11] According to a Cochrane review, use of a fluoride mouth rinse as an adjunct to regular twice daily use of fluoride toothpaste has long been recommended to children of high caries rate with fluoride mouth rinses showing a clear reduction of caries in terms of decayed, missing and filled tooth surfaces.[64] Fluoride containing mouth rinses in children can also reduce MS levels,[65] but they are not recommended for children under 6 years of age due to the risk of fluoride toxicity from acute and chronic fluoride ingestion.[8] Our results are in agreement with previous studies showing that a fluoride mouth rinse

(Neutrafluor 220) has growth inhibitory effects against *S. mutans*, *S. sanguinis* and *L. acidophilus*.

As explained above, pure NaF solutions up to a concentration of 100,000 ppm were ineffective at inhibiting growth of *S. mutans*, *S. sanguinis* and *L. acidophilus*. Hence, in the present study, it is most likely that other compounds in the mouth rinse formulation enhanced the action of NaF or had anti-bacterial effects in their own right. For example, Neutrafluor 220 contains sodium benzoate, a common food preservative with known bacteriostatic properties.[66, 67] Benzoate reduces biofilm thickness and bacterial vitality, though not to the extent of CHX. [66] However, benzoate has not been tested on oral bacteria, and was not tested as part of this study.

PI is available in a mouth rinse formulation and as a topical chair-side treatment for children and adults. Tanzer et al[68] (1977) achieved bactericidal effects on *S. mutans* using inorganic iodine at concentrations as low as 0.04% and showed the selective suppression of *S. mutans* versus *S. sanguinis* at these concentrations. In the present study, the compound PI (iodine with polyvinyl pyrrolidone) was used as it is commonly found as an antiseptic in medicine and dentistry. It has fewer of the problematic side effects compared to pure iodine (staining and mucosal irritation), while still being effective at altering biofilm formation.[69] Our results show comparable inhibition of *S. mutans* and *S. sanguinis*, and thus do not agree with those reported by Tanzer and coworkers.[68]

Previous studies have shown that in-chair topical application of 10% PI is effective at preventing white spot lesions in toddlers when applied every 2 months, even if the child is bottle feeding on cariogenic substrates at naptime.[70, 71] Similarly, a once-only topical application of 10% PI followed by acidulated phosphate fluoride in children produced a prolonged decrease in MS and *Lactobacillus* levels over 3 months.[72, 73] This approach did not, however, prevent new carious lesions from developing over the subsequent year post application, probably due to persistent cariogenic dietary factors and poor oral hygiene.[72, 73] Research by Simratvir and coworkers[74] showed that a 3-monthly topical application of 10% PI following dental restorative treatment can reduce *S. mutans* levels and prevent further occurrence of caries. Our present results are in agreement with the above studies showing PI at concentrations of 10% and higher can inhibit the growth of

S. mutans, S. sanguinis and *L. acidophilus. O*ur results also extend previous studies by showing that a mouth rinse containing 0.1% PI (Betadine) is ineffective at inhibiting the growth of *S. mutans* and *S. sanguinis*.

Mouth rinses containing EO are found in the market and one of the leading brands is Listerine. This commercial product was tested and produced the greatest inhibition on *L. acidophilus* of all mouth rinses tested. On testing the four principal active agents in pure form dissolved in buffer, none of the four agents (menthol, thymol, methyl salicylate and eucalyptol) was effective at inhibiting the growth of *S. mutans*, *S. sanguinis* and *L. acidophilus.* The test concentration of 0.1% was a higher concentration than that found in Listerine, however, there was no surfactant used in the present study, so the bio-availability of these agents would be limited. We avoided the use of a surfactant to dissolve the active agents as the surfactant would have itself exerted anti-bacterial effects. The bactericidal properties of EO have been attributed to their ability to effectively permeate through established plaque leading to inhibition of enzymes.[75] Moreover, the combination of the actives is thought to be synergistic, and this was not tested in the present study. However, conflicting data exists in respect to Listerine's anti-plaque efficacy. Several studies have shown beneficial effects of EO mouth rinses such as significantly reducing total plaque bacterial counts,[76, 77] reducing MS levels in teenagers,[78] and improving plaque and bleeding scores in orthodontic patients[79, 80] while another study has shown negligible anti-bacterial effects.[81] In the assay system used, the zero ethanol Listerine Zero mouth rinse was ineffective at inhibiting bacterial growth. However, we did not test the ability of the rinse or its components to permeate plaque, a key bactericidal property of EO. Our present results suggest that EO do not figure dominantly for prevention of caries in high risk children.

NaOCl is commonly used as a disinfectant for cleaning baby products such as bottles and pacifiers. Disinfecting objects shared between mother and child, such as bottles, cutlery and toothbrushes, with a strong disinfectant is a possible mechanism for reducing the vertical transmission of oral cariogenic bacteria from mother to child. NaOCl has also been suggested as a disinfectant for toothbrushes with promising results for MS reduction.[82, 83] NaOCl is not commonly used as a mouth rinse due to its poor palatability; however, recent studies are reviewing its use for treating adult periodontal disease as

evidence shows it can improve plaque and bleeding scores at concentrations as low as 0.05%.[84, 85]

Our present results disagree with previous results as we found that pure NaOCl at concentrations below 2% gave no bacterial inhibition. In the present study, 2% NaOCl in the form of commercial Milton's solution was ineffective against *S. mutans* and *S. sanguinis*; however pure NaOCl at 2% concentration produced growth inhibition of both *S. mutans* and *S. sanguinis*. Similarly, Milton produced less growth inhibition against *L. acidophilus* compared to a pure 2% NaOCl solution. These results suggest that Milton rinse is less effective compared to pure NaOCl at inhibiting bacterial growth. This could be due to antagonistic effects with other agents within the formulation which were not investigated in this study or the solution reaching its expiry date, and thus having a diminished anti-bacterial activity.

In high caries risk children, another alternative to a high fluoride product is using combinations of agents that are low in individual concentrations, ideally with synergistic anti-bacterial effects. For reasons of safety, the lowest effective concentrations of agents that can be combined with fluoride compounds should be considered for pediatric use. The advantages of combinations with synergistic or additive effects are greater anti-caries and/or anti-plaque effects compared to the individual compounds, whilst reducing dose and therefore enhancing safety. Such strategies for boosting the efficacy and safety of medications are used commonly in medicine and dentistry. For example, the staining and mucosal irritation of pure iodine is reduced by combining iodine with polyvinyl pyrrolidone. [69] However, in contrast to synergistic and additive effects which are usually beneficial, some combinations of antiseptics can result in interference and reduced efficacy .[86] The results of the present study show that a range of interference issues can occur (Table 7). Minimal evidence exists for combinations of antiseptics such as CPC, PI, CHX or NaOCl so these compounds were tested in all combinations, including with NaF. As a detergent commonly found in fluoride dentifrices, SLS was also included as it has already been shown to have growth inhibitory effects.

The results of the present study provide additional support to the notion that PI can form part of a caries preventive program. To date, PI has been used in children for

professional topical applications for control of early childhood caries. The combined use of topical polyvinyl-pyrrolidone-iodine and fluoride varnish can result in more caries-free first permanent molars in children aged 5 to 7 years compared to fluoride varnish alone.[87] Another study by the same research group concluded that a combination of 10% PI and 5% NaF in a varnish reduced the rate of new carious lesions by 31% over a fluoride varnish alone in children aged 1 to 3 years.[88] Initially in this study, 10% PI was combined with 0.1% NaF which produced comparable results to PI alone for all three bacteria. PI and NaF were then combined at concentrations well below those at which the individual compounds are normally used. This produced additive anti-bacterial effects against *S. mutans* and *S. sanguinis,* in agreement with previous studies. Such low dose combinations could have promise for safe use in small children and infants.

Also of interest, NaOCl in combination with PI produced additive anti-bacterial effects against *L. acidophilus.* This combination has not been tested previously as these agents are not commonly used together and NaOCl is only in the early stages of testing for anti-plaque effects.[84, 85] Despite this additive effect against *L. acidophilus*, caution must be used as there was interference between these agents when tested against *S. mutans.*

Overall, of the numerous combinations tested, few produced useful additive anti-bacterial effects over and above those seen from the individual compounds. In most cases, growth inhibition was comparable to the more effective individual compound within the pair used. For example, NaF and CHX produced anti-*S. mutans* effects that were comparable to pure CHX, but greater than that found for NaF alone. These results agree with previous data where varnish and gel formulations showed a useful reduction of *S. mutans* levels and increased enamel mineral density.[89-91]

In fact, many combinations tested showed interference and reduced efficacy. The results of the present study show that if chlorhexidine (0.01%) is combined with sodium lauryl sulfate (0.25%) or sodium hypochlorite (0.5%) with povidone iodine (10%), or cetyl pyridinium chloride (0.01%) with sodium fluoride (0.1%), the inhibitory activities against *S. mutans* and *S. sanguinis* of the primary compounds will be reduced. These agents are, therefore, not ideal for use in a single anti-bacterial product.

CPC combined with NaF is one such combination that showed interference against both *S. mutans* and *S. sanguinis.* This is in agreement with the known interactions of this molecule with several studies showing antagonism between fluoride toothpaste and CPC mouth rinse use.[92, 93]

Similarly, it is well known that toothpastes containing SLS can impair the antimicrobial effects and substantivity of CHX due to formation of an insoluble salt following the reaction between CHX (a cation) and SLS (an anion).[94] One clinical investigation has showed that rinsing with a 1.5% SLS slurry prior to using a 0.2% CHX rinse reduced the anti-plaque efficacy of the CHX. [95] The results of the present study are in agreement with these findings as SLS interfered with the anti-bacterial properties of CHX against both *S. mutans* and *S. sanguinis* .

The current results also show differences in the responses of individual bacterial species. *L. acidophilus* was inhibited by all the combinations tested, whereas *S. mutans* and *S. sanguinis* were inhibited by most but not all combinations. The comparable effects observed with *S. mutans* and *S. sanguinis* likely reflect the fact that the two *Streptococcal* species have general similar cell wall structural properties.[41] On the other hand, the combinations of CPC + PI and PI +SLS elicited interference on *S. sanguinis* but not on *S. mutans*, suggesting that some differences exist between these bacteria that can lead to different responses with different combinations of antiseptics.[96, 97] Similarly, cell wall and metabolic differences between the streptococci and lactobacilli can cause differences in responses of the bacteria.[98, 99] Although the exact roles of these bacteria in the caries process are not clear, it is generally thought that *S. mutans* is actively involved in the caries process while lactobacilli are likely to be secondary invaders.[100, 101] *S. sanguinis*, on the other hand is thought to exist in competition with *S. mutans* in the dental biofilm, and children with caries have less *S. sanguinis* and more *S. mutans* while the reverse is true for children without caries.[102, 103]

Based on these concepts, the combinations of antiseptics that selectively inhibit *S. mutans*, but not *S. sanguinis* nor lactobacilli would be desirable for removal of *S. mutans* without affecting other plaque bacteria. The combination CPC+ PI meets this criteria as it interfered with the inhibition of *S. sanguinis* and *L. acidophilus* without affecting the

inhibitory effects on *S. mutans* of the individual compounds. In contrast, the combination of PI + SLS, while it interferes with inhibition of *S. sanguinis*, does not alter the inhibition of *L. acidophilus* by the individual compounds.

Although this study is limited by the testing of single bacterial cultures under neutral pH conditions rather than in complex biofilms under varying pH conditions, the results add to the understanding of the antimicrobial effects of the test compounds individually and in combinations.[86] The present results do not, however, indicate the clinical effectiveness of the agents.[86] This is due to the nature of the plaque biofilm and the protection provided by the extracellular matrix which elevates the minimum inhibitory concentrations required for testing biofilms versus planktonic cells.[86] Furthermore, saliva plays an important role in the dilution and clearance of compounds from the oral cavity, and it may interact with antiseptic agents.[86] Saliva also affects the diffusivity of the agents, reducing the concentration of agents and hence their inhibitory effects. In the agar diffusion system, the dentifrices likely reacted differently to the individual compounds in solution due to their differences in ability to diffuse across the agar system. Once again, this is not a good representation of the intraoral interactions of these compounds. Hence, clinical trials are necessary to elucidate further the interactions between the antiseptic compounds, saliva and the bacterial biofilm.

A further and final point is that the agar diffusion method employed in this study measured only the growth inhibition of the bacteria under neutral pH conditions. It is possible that the individual compounds and combinations tested could possess other mechanisms of anti-bacterial activity at neutral or at other pH values such as interference with adherence to the tooth surface or to other bacteria which could not be investigated in the present assay system. For example, fluoride reduces caries largely through remineralisation mechanisms rather than anti-bacterial activity. The ability for fluoride to promote remineralisation may in fact be more important for disease control than its anti-bacterial effects. As such, these results should be analysed cautiously, taking into account this study's limitations. On the other hand, inhibiting the growth of *S. mutans* in young children during the early stages of colonisation may prevent its long term establishment in the oral cavity. To this end, tooth brushing with paste containing

compounds with anti-*S. mutans* activity can contribute to the prevention of early childhood caries.

Recommendations for future research

This research has isolated several compounds and combinations of compounds that have growth inhibitory effects against both mutans and non-mutans bacteria. The pure agents chlorhexidine, triclosan and sodium lauryl sulphate, are already available on the market in toothpaste and mouth rinse formulations. However, this research has shown that lower concentrations of these compounds can have significant bacterial growth inhibitory effects. As such, further research should be undertaken to find a formulation with low concentration of active ingredients with maximal inhibitory effect.

Also of note, the agents tested have other modes of action besides the ability to inhibit bacterial growth. For example, chlorhexidine at high concentrations is bactericidal causing direct cellular effects through precipitation of intracellular cytoplasm and impeding the action of glucosyltransferase thus preventing adhesion of bacteria to the tooth's surface. Further testing of these other modes of action should be undertaken to determine the minimum concentration required to create such effects. The formulation of a product suitable for children requires the balancing of a low concentration active agent while maximising the anti-bacterial effects.

The combinations found to have additive growth inhibitory effects were povidone iodine with sodium fluoride and cetyl pyridinium chloride with sodium lauryl sulphate. Initial in vitro testing of experimental products containing povidone iodine with sodium fluoride has been undertaken but further testing in other formulations is required. In vitro studies of products containing both cetyl pyridinium chloride and sodium lauryl sulphate in a single agent has not been undertaken and requires further research. It must also be determined that the active ingredients are compatible with the abrasive system or surfactant in the paste or rinse formulation.

Following the in vitro testing of these agents and combinations of compounds, clinical trials are required to determine the effect of these agents on a variable plaque mass, comparing the effects on salivary versus plaque bacteria and taking into account patient compliance. These agents show great promise for use by children due to their growth inhibitory effects at low concentrations. With further research, a new low concentration

formulation may be produced which is suitable for the prevention of early childhood caries.

Conclusions

In conclusion, this research showed that children's toothpaste can inhibit the growth of both mutans and non-mutans bacteria, although the inhibition is comparatively lower compared to standard toothpastes containing 1450 ppm F. Pure sodium fluoride and sodium monofluorophosphate did not produce growth inhibitory effects against *S. mutans, S. sanguinis* or *L. acidophilus.* Further testing of compounds within the toothpaste formulations revealed sodium lauryl sulphate and triclosan at concentrations found in toothpastes inhibited bacterial growth. Other common components of toothpaste, such as polyethylene glycol and pyrophosphate at concentrations as high as 8000 ppm, did not show bacterial growth inhibitory effects.

Common antiseptics at low concentrations have potential to inhibit the growth of cariogenic bacteria. Of the agents tested, chlorhexidine produced the greatest growth inhibition against *S. mutans*, *S. sanguinis* and *L. acidophilus* at the lowest concentration.

Various combinations of antiseptics and sodium fluoride solutions at low concentrations have potential to decrease numbers of cariogenic bacteria and may be suitable as an adjunct agent for reducing risk for early childhood caries. Of the agents tested, povidone iodine with sodium hypochlorite produces additive growth inhibition against *L. acidophilus* while povidone iodine with sodium fluoride produced synergistic growth inhibition against *S. mutans* and *S. sanguinis.*

These compounds isolated as having growth inhibitory effects against mutans bacteria may be suitable for use by children, especially those at high risk for early childhood caries. Further testing of these compounds at low concentrations in vitro and in clinical settings is necessary to determine if combinations of these agents may be suitable for use in children.

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