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

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ABSTRACT

Background: Oral antiseptics are valuable in controlling oral infections caused by cariogenic bacteria. The aim of this study was to investigate the effects of mouthrinses 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 mouthrinses 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 mouthwash produced the greatest mean growth inhibition of *S. sanguinis* and *S. mutans* compared to all other mouthrinses 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*.

Keywords: Antiseptics, chlorhexidine, mouthrinses, *Streptococcus mutans*.

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INTRODUCTION

Caries in children is initiated by colonization 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 colonized 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 mouthrinses 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 toothbrushing.

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 mouthrinse 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 minimize 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 mouthrinses 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 mouthrinses 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 mouthrinses and pure antiseptic compounds on *S. mutans* and non-mutans bacteria, *S. sanguinis* and *L. acidophilus* using mono-bacterial cultures.

MATERIALS AND METHODS

Commercially available antiseptic mouthrinses 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 mouthrinses as there were no alcohol-free formulations available commercially. The test mouthrinses 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 mouthrinses 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). *Streptococcus mutans* ATCC 25175 and *S. sanguinis* ATCC 10556 were revived from freeze-dried vials. *Lactobacillus 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 37 °C. 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 mouthrinse 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 0.03 g. Sixty microlitres of mouthrinse or pure compound was pipetted into each well containing the filter paper.

Five millilitre aliquots of a bacterial suspension (6 x 10^8 bacteria/mL) were added to 5 mL of melted Muller-Hinton agar at 45 °C, then mixed thoroughly and poured evenly over the surface of each agar plate

Table 1. Active ingredients found in antiseptic mouthrinses commercially available in Australia

Mouthrinse 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%)

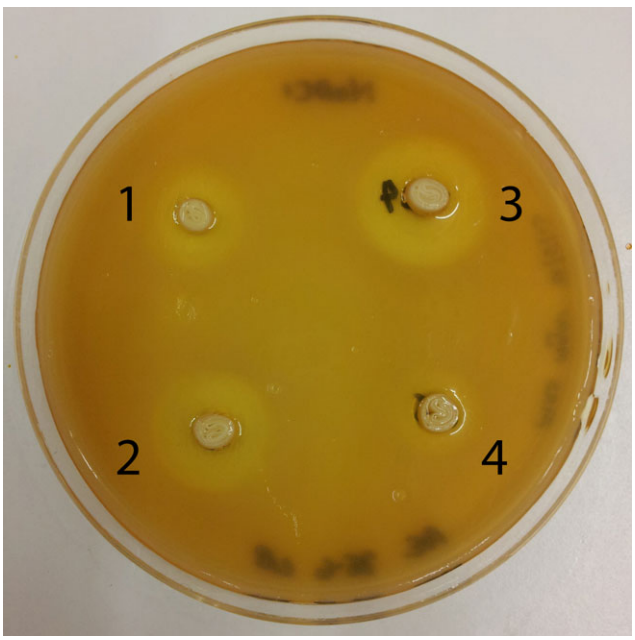

Fig. 1 Photograph of a microbial culture plate with wells containing sodium hypochlorite at various concentrations. Well No. 1 contained a pure solution of 1% sodium hypochlorite, Well No. 2 a pure solution of 4% sodium hypochlorite, Well No. 3 a pure solution of 2% sodium hypochlorite, and Well No. 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.*

containing wells loaded with test medicaments.[16] The culture plates were inverted and incubated at 37 °C for 72 hours. 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 (Fig. 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 mouthrinses, 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 mouthrinses 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 mouthrinses 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 $p < 0.01$).

Lactobacillus acidophilus was inhibited by all mouthrinses 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. *Streptococcus sanguinis* underwent the greatest growth inhibition from 11.2 ± 0.86 mm at 0.01% to 21.8 ± 0.75 mm at 0.8%. *Lactobacillus acidophilus*

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

Mouthrinse	Active compound (concentration %)	Zone of inhibition mean diameter (mm ± SD)			n	*p*-value	
		S. mutans (SM)	*S. sanguinis* (SS)	*L. acidophilus* (LB)		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*

Chlorhexidine (%wt/vol)	Zone of inhibition mean diameter (mm ± SD)			n	*p*-value	
	S. mutans (SM)	*S. sanguinis* (SS)	*L. acidophilus* (LB)		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).

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

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

Sodium hypochlorite (%vol/vol)	Zone of inhibition mean diameter (mm ± SD)			n	*p*-value	
	S. mutans (SM)	*S. sanguinis* (SS)	*L. acidophilus* (LB)		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*

Povidone iodine (%wt/vol)	Zone of inhibition mean diameter (mm ± SD)			n	*p*-value	
	S. mutans (SM)	*S. sanguinis* (SS)	*L. acidophilus* (LB)		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).

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 mouthrinse. 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 chlorhexi-

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

	Zone of inhibition mean diameter (mm ± SD)				*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).

dine, 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 anticaries agent, although studies have demonstrated that pre-surgical 0.05% cetylpyridinium chloride mouthrinses by children aged 10–15 years are efficacious at reducing both aerobic and anaerobic microorganisms.[28] 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 mouthrinse (Neutrafluor 220) has growth inhibiting effects against *S. mutans*, *S. sanguinis* and *L. acidophilus*.[29–31] In the present study, it is most likely that other antibacterial compounds in the mouthrinse formulation enhanced the action of sodium fluoride. As Neutrafluor 220 contains sodium benzoate, a common food preservative with known bacteriostatic properties,[32] 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 mouthrinse 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 are 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.[33,34] 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.[10,35]

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.[36] 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.[36]

Mouthrinses 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 mouthrinses 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.[37]

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 mouthrinses such as significantly reducing total plaque bacterial counts,[14] reducing mutans streptococci levels in teenagers,[38] and improving plaque and bleeding scores in orthodontic patients.[39] In our assays, Listerine Zero (no ethanol) mouthrinse 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 colonization of cariogenic bacteria in children.[40] Although it has poor palatability as a mouthrinse, 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%.[41,42] 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 the 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 microbiologcal 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 persister 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 mouthrinse.

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