Effects of Chlorhexidine, Listerine and Fluoride Listerine Mouthrinses on Four Putative Root-caries Pathogens in the Biofilm

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Objective: *To investigate the effects of chlorhexidine (CHX), Listerine and Fluoride Listerine on putative root-caries pathogens in the biofilm in the artificial mouth model.*
Methods: *A total of 24 human dentine discs were prepared. A biofilm composed of* Streptococcus mutans*,* Streptococcus sobrinus*,* Lactobacillus rhamnosus *and* Actinomyces naeslundii *was cultured on the surfaces of human dentine discs in an artificial-mouth model. Sucrose was supplied by computer-controlled release on a daily basis to simulate the real-life situation. Three treatment reagents, CHX, Listerine and Fluoride Listerine, were supplied at a flow rate of 15 ml/h for 6 min twice a day. The dentine discs with biofilm were removed from the artificial mouth after being cultured for 1, 2, 3, 4, 5 and 6 days. The bacteria in the biofilm were analysed by plating on BHIS agar and the colony-forming units of each species were counted.*
Results: *The total number of bacteria in the CHX group was significantly lower than in the other three groups (including control). There was no decline in the number of bacteria in the Listerine group.* S. mutans *was reduced significantly in the CHX group compared with the control group. The number and proportion of* A. naeslundii *in the CHX group were significantly lower than in the other three groups. The proportion of* L. rhamnosus *in the CHX group was significantly higher than in the other three groups.*
Conclusion: *CHX has the most significant effect on inhibition of the putative root-caries bacteria, with the exception of* L. rhamnosus*. Both Listerine and a combination of fluoride and Listerine could not effectively reduce the numbers of bacteria in the biofilm. The effects of CHX, Listerine and Fluoride Listerine on root caries prevention need further investigation.*

Key words: *artificial mouth, mouthrinse, oral biofilm, root caries*

Bacteria in plaque biofilm metabolise carbohydrates to organic acids that cause dental caries. Laboratory models of this process using the artificial mouth model are potentially valuable in understanding the mechanisms involved and for developing procedures[1-2]. Conditions within the mouth change with time and are difficult to control and manipulate[3-4]. Thus, *in vitro* studies offer advantages, because most of the environmental conditions and the microbiota can be controlled.

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It has been shown that bacterial cells in biofilm are more resistant to antimicrobials than bacterial cells in suspensions[5-6]. A number of biofilm models have been developed to evaluate the effects of caries-preventive agents on caries pathogenic bacteria[7-8]. Characteristics of biofilms formed by four putative root-caries pathogens, *Streptococcus mutans*, *Streptococcus sobrinus*, *Lactobacillus rhamnosus* and *Actinomyces naeslundii*, in the artificial mouth have been used for studies of root caries[9].

Mouthrinses containing different antibiotics have been used to inhibit cariogenic bacteria in plaque. Among them, chlorhexidine (CHX) is a chemical antimicrobial that kills both Gram-negative and Gram-positive microbes by attacking the bacterial cytoplasmic or inner membrane. It has been shown that CHX application is efficacious in inhibiting growth of *S. mutans*

Table 1 Compositon of Corsodyl, Listerine and Fluoride Listerine used

	Compositon (W/V)	Manufacturer
Corsodyl	Chlorhexidine digluconate 0.2%	SmithKline Beecham Consumer Healthcare, Maidenhead, UK
Listerine	Thymol 0.06%, eucalyptol 0.09%, methyl salicylate 0.06%, menthol 0.04%, ethanol 26.7%, benzoic acid 0.15%, water 100%	IDS Manufacturing, Thailand
Fluoride Listerine	Thymol 0.064%, eucalyptol 0.092%, methyl salicylate 0.06%, menthol 0.043%, benzoic acid 0.15%, sodium fluoride 0.0221%, water 100%	IDS Manufacturing, Thailand

and retarding acid production by cariogenic bacteria[10-12]. CHX has been used in mouthrinses, gels and varnishes, but its caries-prevention effect is still inconclusive[13]. Few studies have reported its effects on root caries. Antimicrobial essential oil mouthrinses, such as Listerine, can be accessed over the counter. Essential oil can kill microorganisms by disrupting their cell walls and inhibiting enzyme activities. It can prevent bacteria from aggregating with Gram-positive pioneer species and extracts endotoxins from Gram-negative pathogens[14]. Additionally, it may reduce the bacterial load, slow down plaque maturation and decrease the plaque mass and its pathogenicity[15]. Other reports have shown that essential oil can reduce numbers of *S. mutans* in saliva[16]. Listerine can also inhibit the *S. mutans* biofilm formation *in vitro*[17], and may have a role in preventing the development of caries[18].

Fluoride has well-known caries-preventative effects[19]. A pilot study using combined 0.2% CHX gluconate and 0.055% sodium fluoride as a mouthrinse showed that the mineral gain of bovine teeth for this CHX/fluoride mouthrinse was significantly higher than for both the CHX and placebo treatment groups[10]. Similarly, fluoride combined with an essential oil mouthrinse may also have a better preventive effect on caries.

The aim of the present study was to investigate the effect of three antimicrobial mouthrinses, comprising CHX (Corsodyl), Listerine and Fluoride Listerine, on biofilm composed of four species of cariogenic bacteria (*S. mutans*, *S. sobrinus*, *L. rhamnosus* and *A. naeslundii)* on human dentine surfaces in an artificial-mouth model.

Materials and methods

Formation of oral biofilm in the artificial mouth

An artificial saliva buffer (defined medium mucin, DMM) was prepared as described by Shellis[20], and the growth media for the *in vitro* plaque assay was prepared with a 50% saliva-based medium.

Sound human third molars were collected from Peking University School and Hospital of Stomatology, Beijing, China. Human tissue usage consents were obtained from the patients. Dentine disks of 5 mm diameter were prepared across the cementoenamel junction. The dentine discs were put into four disc holders, each holding six specimens. The non-experimental surfaces of the disks were covered with clear nail varnish to prevent microleakage of oral biofilm. After varnish application, the dentine disks were autoclaved, remaining in sterile human saliva for 3.5 h to allow formation of the pellicle[21]. Then, 5 ml of an overnight culture of the four species of bacteria with 15 ml of fresh medium was added to the 24 dentine discs and cultured anaerobically for 1 day to allow the bacteria to settle on the experimental surfaces. After that, all the dentine discs were put in the artificial mouth. The four groups of disks were cultured using *(1)* distilled water; *(2)* Listerine; *(3)* Fluoride Listerine; and *(4)* CHX (Corsodyl), see Table 1. The total sample size was 24, with 6 disks in each group.

Artificial mouth set-up

The parts of the artificial mouth equipment and accessories were autoclaved before use and the laboratory was irradiated with ultraviolet light for 2 h prior to the experiment. The experiment set-up was carried out under aseptic conditions[22].

Media supply: sucrose (5%) was supplied by computer control every 8 h for 6 min with a flow rate of 15 ml/h to simulate the real-life eating situation. Simulated oral fluids (DMM) were continuously supplied at 3.6 ml/h. The four treatment reagents were supplied at a flow rate of 15 ml/h for 6 min twice a day (1 h after sucrose)[23] (see Fig 1).

After culture for 2 days in the artificial mouth, the first dentine disc from each group was taken out for

Table 2 The numbers and proportions of bacteria in the biofilm of each group: lg(CFU/ml+1)/%

	Control	Chlorhexidine	Listerine	F Listerine
Total bacteria	6.6065	5.3744*	6.6772	6.5350
S. mutans	5.1372/8.31	2.3239*/15.66	4.2692/8.79	3.5102/14.78
S. sobrinus	4.3729/19.60	2.4669*/9.68	3.3010/2.39	5.2919/10.97
A. naeslundii	6.1524/53.84	2.0214*/13.80*	6.6139/88.06	6.4011/76.23
L. rhamnosus	1.9450/2.73	4.1765/50.86*	3.8011/0.74	3.7479/9.23

*ANOVA test, ($P < 0.05$)

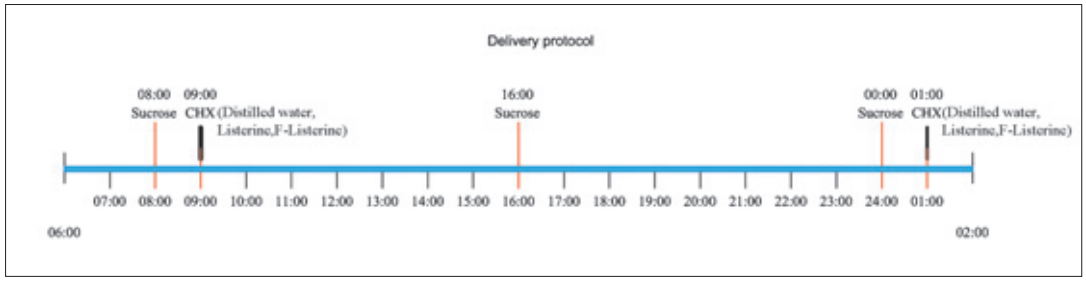

Fig 1 Delivery timeline used in the experiment.

analysis. The second disc was taken out after 3 days, then the third one after 4 days. Finally, the last one was taken out after 6 days.

Assessment of biofilm viability

The biofilm was collected by using autoclaved spatulas and transfered to phosphate buffered saline (PBS) solution. The PBS was serially diluted and inoculated on the blood agar. The agars were cultured anaerobically for 5 days. At the end of day 5, the bacterial compositions of the biofilm were analysed. Bacteria were identified by colony morphology, Gram stain and microbiological counts under a confocal laser scanning microscope (Olympus FluoView™ FV1000, Hong Kong) to confirm there was no contamination. Colony-forming unit (CFU) counts were recorded.

Statistical analysis

SPSS software (version 10.0, SPSS, Chicago, IL, USA) was used for the statistical analysis. Means, standard deviations and confidence intervals (95% CI) were determined for all data. The total bacteria, *S. mutans*, *S. sobrinus*, *A. naeslundii* and *L. rhamnosus* levels in CFU/ml of biofilm were converted into log values [lg(CFU/ml+1)] for statistical analysis. ANOVA and Mann–Whitney non-parametric analysis were used to compare the bacteriological counts and proportions of each species in the biofilms.

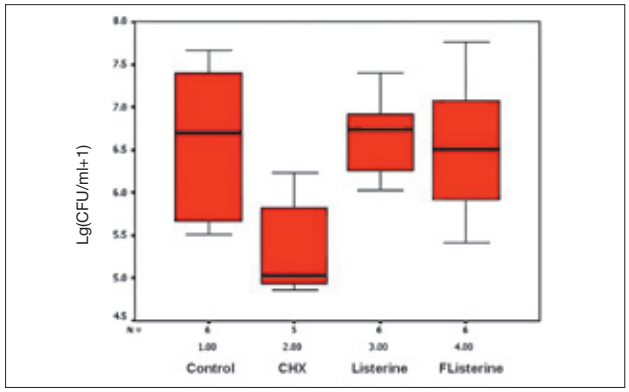

Fig 2 The total numbers of bacteria in control, CHX, Listerine and Fluoride Listerine groups.

Results

Under the confocal laser scanning microscope, no contamination by other bacteria was observed.

The total number of bacteria in the CHX-treated group (5.3744) was significantly lower than in the other three groups ($P < 0.05$) (Fig 2). The number of *S. mutans* in the CHX group (2.3239) was significantly lower than in the control group (5.1372) ($P < 0.05$). The

number (2.0214) and proportion (13.80%) of *A. naeslundii* in the CHX group was significantly lower than in the other three groups ($P < 0.05$). The number of *S. sobrinus* in the CHX group (2.4669) was significantly lower than in the Fluoride Listerine group (5.2919) ($P < 0.05$) (Table 2).

There was no reduction in the total numbers of bacteria in the biofilm in the Listerine group (6.6772) and the Fluoride Listerine group (6.5350). The proportion of *A. naeslundii* in the Listerine group (88.06%) was significantly higher than in the control group (53.84%) and CHX group (13.80%) ($P < 0.05$).

There was no difference in the numbers of *L. rhamnosus* between the four groups ($P > 0.05$), while the proportion of *L. rhamnosus* in CHX group (50.86%) was significantly greater than in the other three groups ($P < 0.05$).

Discussion

Biofilm models are important tools for evaluating the biochemical and microbiological composition of biofilms formed under different conditions. In the present study, biofilm composed of the four species of bacteria that are most closely associated with root caries was cultured on human dentine surfaces[9], in conditions that closely simulated the situation on the human root surface. Sugar was added to the biofilm by computer control to simulate real-life conditions, while at the same time different antibiotic mouthrinses were used to treat the biofilm. The results were intended to shed further light on the effect of these mouthrinses. Furthermore, as bacteria are more resistant to antibiotics when in the form of biofilm than in planktonic form[6], investigation of the effect of mouthrinses on biofilm will be helpful for simulating their effects *in vivo*.

CHX is a broad-spectrum antimicrobial agent, effective against streptococci, *Actinomyces*, Gram-negative rods, yeasts, total aerobes and total anaerobes. High concentrations of CHX have an immediate bactericidal effect, penetrating the bacterial cell wall and leading to precipitation of the cytoplasm[24], whereas lower concentrations, such as 0.012% CHX, are bacteriostatic. In the present study, CHX significantly inhibited biofilm growth, with the total numbers of bacteria in the biofilm treated with CHX being reduced significantly; the numbers of *S. mutans*, *A. naeslundii* and *S. sobrinus* were also reduced significantly. In particular, the proportion of *A. naeslundii* in the CHX-treated group was significantly lower than in the other three groups, which suggests *A. naeslundii* might be more sensitive to CHX.

It is well known that *S. mutans* and *S. sobrinus* are the most important cariogenic bacteria. Although CHX could inhibit *S. mutans* and *S. sobrinus*, it is not necessarily an effective way to prevent caries[13]. Autio-Gold thought that the effectiveness of CHX in reducing the levels of *S. mutans* and in plaque reduction might not always correlate with eventual caries reduction.[13] Another study showed that *S. mutans* levels would increase again if CHX use is stopped for a period of time[25]. Some others suggested that other bacteria would be more sensitive to CHX than *S. mutans* and *L. rhamnosus*, and therefore an overgrowth of *S. mutans* and *L. rhamnosus* could occur[26].

In the present study, the number of *L. rhamnosus* in the CHX group (4.1765) was higher than the number in the control group (1.9450), and the proportion was significantly higher than in the other three groups. This may support the view that *L. rhamnosus* is not sensitive to CHX[27]. Another possible explanation might be that without mouthrinse treatment the other three species could outgrow *L. rhamnosus* in the multispecies biofilm, and that *L. rhamnosus* was therefore inhibited by the other bacteria in the control group.

An *in vivo* investigation using CHX in gel and varnish forms has proven CHX to be effective in *S. mutans* reduction in both saliva and dental plaque[28]. Other *in vivo* research has shown that CHX can decrease the amount of supergingival plaque and inhibit the formation of plaque[29]. One-year placebo-controlled randomised trials have demonstrated a significant reduction and control of root caries lesions, suggesting regular antibacterial applications to be beneficial for those patients with xerostomia[30].

Further research should be undertaken to confirm the effect of CHX in the prevention of root caries. Importantly, it should be kept in mind that CHX is a broad-spectrum antimicrobial agent and so has an antibiotic effect on most bacteria in the oral cavity, therefore its use might risk disturbing the microecological balance.

The main component of Listerine is essential oil, which has been reported to be antiseptic. *In vivo* research has found that essential oil can reduce levels of plaque and inhibit plaque formation[31]. Other research found that Listerine and CHX-containing mouthrinses showed equivalent antimicrobial activity when used on biofilm from human saliva[32]. Numerous clinical studies have confirmed the plaque-inhibitory properties of Listerine. Twice-daily application of Listerine mouthrinse suppressed *de novo* plaque formation after 4 days[31] and after 21days in the absence of mechanical toothcleaning[33-34]. The essential oil mouthrinse produced reductions in total recoverable streptococci and in *S. mutans* and other anaerobes in plaque, and corresponding reductions in saliva[16,35].

In the present study, no difference in the total numbers of bacteria could be seen in the Listerine group and control group. Listerine did not inhibit the growth of the four species in biofilm. The proportion of *A. naeslundii* in the Listerine group was significantly higher than in the control and CHX groups. Brecx reported there was no evidence of Listerine having an antibacterial effect *in vivo*[34]. Listerine also could not effectively inhibit *A. naeslundii* and *S. mutans in vitro*[36].

Another report showed that the essential oils inhibited the adhesion of *S. mutans* and decreased the amount of plaque[37]. The present authors speculate that in this study the bacteria had already settled down on the surface of the dentine discs, so Listerine could not have reduced the number of bacteria by affecting their adhesion.

One *in vivo* study showed that after Listerine treatment, dental plaque produced less lactate and less acetate. Listerine was effective in reducing plaque acidogenicity[19]. However, as Listerine did not inhibit the growth of biofilm in the present study, there was no suggestion that Listerine could reduce acid production. Further studies should be carried out to determine whether Listerine could be used to prevent root caries.

It has been well accepted that fluoride can prevent caries by inhibiting bacteria and inducing remineralisation. In the present study, the number of bacteria in the group treated with Fluoride Listerine was lower than in the Listerine group, but the difference was not statistically significant. Fluoride could not inhibit the growth of bacteria at the concentration in the mouthrinse (0.022%). It has been reported that even 0.05% fluoride could not reduce the number of viable bacteria in *S. mutans* biofilm[38]. Some other studies have reported that although fluoride did not exhibit any antimicrobial effect, fluoride could inhibit the demineralisation of the enamel surface and induce its remineralisation, which could cause a reduction in the amount of new caries. The main effect of fluoride in caries prevention was physicochemical[38-40]. Fluoride Listerine may have a better caries prevention effect than Listerine.

Conclusion

Of the mouthrinses included in this study, CHX was the most effective at inhibiting putative root-caries bacteria in the biofilm. However, the use of CHX as an antiseptic agent for the prevention of root caries remains controversial. Both Listerine and a combination of fluoride and Listerine did not effectively reduce the numbers of bacteria in the biofilm.

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