**Abstract:** Mouth rinses are a useful supplementary tool for the prevention of oral infectious diseases. Although the antimicrobial effects of mouth rinses have been investigated, there are few studies focusing on the comparison of the effects among various oral bacterial species. In the present study, the inhibitory effect of a commercial mouth rinse, “ConCoolF,” and each of its major components, chlorhexidine gluconate, ethanol, and green tea extract, on multiple species of oral bacteria was investigated. Inhibition of bacterial growth was observed in all cariogenic streptococcal species with different genera, serotypes, and strains isolated from different countries when either the complete mouth rinse or chlorhexidine gluconate were exposed to ethanol or green tea extract. Interestingly, growth inhibition was greatly reduced in non-cariogenic streptococci compared with cariogenic streptococci. In addition, both the mouth rinse and chlorhexidine gluconate inhibited the biofilms formed by both Streptococcus mutans (S. mutans) and Porphyromonas gingivalis (P. gingivalis), among which the inhibitory effect against S. mutans was higher than that against P. gingivalis. These results suggest that a mouth rinse containing chlorhexidine gluconate, ethanol, and green tea extract, or chlorhexidine gluconate alone, exhibits antimicrobial activity against several oral bacteria species, having greater activity against pathogenic bacteria.

**Keywords:** antimicrobial effect, chlorhexidine gluconate, mouth rinse, mutans streptococci, Porphyromonas gingivalis

**Introduction**

Dental caries and periodontal diseases are the most common infectious diseases induced by oral pathogens [1]. Multiple agents with antimicrobial properties have been developed and combined to create mouth rinses [2]. Chlorhexidine gluconate, ethanol, and green tea extracts are the major antimicrobial agents included in mouth rinses developed for the purpose of preventing oral diseases [2,3]. Although the antimicrobial effects of these agents against well-known bacteria, such as type strains, have been well established, it is unknown whether these agents have such antimicrobial activity on uncommon oral bacteria, such as those classified into minor serotypes, those that have obtained specific virulence genes, or bacteria identified by newer molecular techniques.

Oral streptococci are known to be a major component in the microbial flora of the oral cavity [4]. Among the oral streptococci, Streptococcus mutans (S. mutans) and Streptococcus sobrinus (S. sobrinus) are classified as mutans streptococci. Mutans streptococci have been implicated as causative agents of dental caries [5]. S. mutans is serologically classified into four groups: c, e, f, and k [6], whereas S. sobrinus is serologically classified into two groups: d and g [7]. Although the distribution frequencies of the serotype f and k S. mutans are less than 3% in the oral cavity, these strains are considered to be associated with cardiovascular disease, such as infective endocarditis and intracerebral hemorrhage [8,9]. Other oral streptococci such as Streptococcus oralis (S. oralis), Streptococcus gordonii (S. gordonii), Streptococcus mitis (S. mitis), and Streptococcus salivarius (S. salivarius) have low cariogenicity, and several of these are regarded as early colonizers of tooth surfaces [10,11].

Periodontal disease is induced by infection with bacterial species associated with periodontitis [12]. Porphyromonas gingivalis (P. gingivalis) is an important periodontal pathogen and is also associated with the development of systemic diseases [13]. Many virulence factors have been reported for P. gingivalis and the virulence of P. gingivalis is highly dependent on its fimA genotype [14]. The efficacy of mouth rinses on P. gingivalis strains with different fimA genotypes has not yet been reported.

In the present study, the ability of a mouth rinse formulated with chlorhexidine gluconate, ethanol, and green tea extract, as well as each individual component, to inhibit the growth of major oral pathogenic bacterial species with different serotypes or genotypes and commensal bacteria with low virulence, were analyzed. In addition, the antimicrobial effects of the mouth rinse or chlorhexidine gluconate on bacterial biofilm formation by two species, S. mutans and P. gingivalis, were compared.

**Materials and Methods**

**Preparation of mouth rinse and antimicrobial agents**

Mouth rinse formulated with chlorhexidine gluconate, ethanol, and green tea extract (ConCoolF) was provided by Weltec Corporation (Osaka, Japan). In addition to the antimicrobial agents, the mouth rinse was composed of monoammonium glycyrrhizinate, L-methanol, propylene glycol, polyoxyethylene hardened castor oil and flavoring agent. The concentrations of the antimicrobial agents were as follows: chlorhexidine gluconate = 0.05%, ethanol = 14.7%, and green tea extract = 0.40%. ConCoolF mouth rinse was diluted into each bacterial broth and tested at concentrations of 0%, 0.001%, 0.01%, 0.1%, 1%, and 10% for use in bacterial growth and biofilm studies. Chlorhexidine gluconate, ethanol, and green tea extract were diluted individually into each broth and tested at concentrations of 0%, 0.001%, 0.01%, 0.1%, 1%, and 10%. A concentration of 100% for each mouth rinse component was defined as the percentage of each agent in ConCoolF, for example, a final concentration of 14.7% ethanol in the broth culture was considered 100% ethanol.

**Bacterial strains and growth conditions**

Table 1 lists the bacterial strains used in this study. A total of six S. mutans strains with different serotypes (two each of serotypes c and k, one each of serotypes e and f) and isolated from different locations in the body (five strains isolated from the oral cavity and one strain isolated from blood) were selected from the laboratory stock [6,15-17]. Among the S. mutans strains, four (TLJ1-1, TLJ10-5, TLJ34-4, and TLJ11-2) were isolated in Thailand and two (MTR148 and TW295) were isolated in Japan. Two S. sobrinus strains from the laboratory stock were used, one each of serotypes d and g [18]. The non-cariogenic streptococci used in this study were S. oralis (ATCC 10557), S. gordonii (ATCC 10558), S. mitis (ATCC 49456) and S. salivarius (HHT), selected from the laboratory stock [18,19]. S. mutans and S. sobrinus strains were cultured on Mitis Salivarius (MS) agar (Difco Laboratories, Detroit, MI, USA) plates containing bacitracin (0.2 U/mL; Sigma-Aldrich Co., St. Louis, MO, USA) and 15% (w/v) sucrose and other
oral streptococci were cultured on MS agar plates. Colonies of each strain were inoculated into brain heart infusion broth (BHI; Difco Laboratories) and cultured at 37°C for 18 h for use in subsequent studies. The P. gingivalis strains used in this study were ATCC 33277 (type I FimA) and OMZ314 (type II FimA), which are known as low- and high-virulence strains, respectively [20]. P. gingivalis was grown in trypticase soy broth supplemented with yeast extract (1 mg/mL), menadione (1 μg/mL), and hemin (5 μg/mL), as previously described [21].

Growth inhibition assay

Growth inhibition assays were performed in accordance with previously described methods, with some modifications [22]. Bacterial cells were collected from broth cultures by centrifugation. Bacteria were resuspended in broth containing the indicated concentrations of mouth rinse or individual antimicrobial agents and adjusted to 1 × 10^7 CFU/mL. After incubation for 18 h at 37°C, bacterial growth was measured using the OD₆₀₀ values. Growth rates were calculated; 100% growth was defined as the growth of each strain in the absence of mouth rinse or antimicrobial agents.

Microscopic observation of in vitro biofilms

The effects of the mouth rinse or antimicrobial agents on the accumulated biofilm structure produced by S. mutans were assessed using confocal laser scanning microscopy, as previously described, with some modifications [23]. Human saliva collected from a healthy volunteer was centrifuged at 12,000 g for 10 min and the supernatant was filtered (pore size: 0.45 μm). The supranatant was diluted 1:3 with Milli-Q water to produce 25% saliva and used to coat 96-well polystyrene microtiter plates. After incubation at 37°C for 2 h, the plates were washed three times with phosphate-buffered saline (PBS). Cultures of each bacterium were adjusted to 0.1 at OD₆₀₀ in a chemically defined medium in the presence or absence of mouth rinse or individual antimicrobial agents at the indicated concentration. Bacteria were then stained with hexidium iodide (15 µg/mL; Molecular Probes, Eugene, OR, USA). The organisms were anaerobically cultured with rocking in saliva-coated wells of a chambered cover glass system (CultureWel, Grace Bio Labs, Bend, OR, USA) at 37°C for 18 h in the dark. Non-attached bacteria were removed by washing with PBS and biofilms were observed by confocal scanning laser microscopy using a TCS SP5 microscope (Leica Microsystems GmbH, Wetzlar, Germany) with reflected laser light at 488 nm, as well as a DMI6000 B fluorescence microscope (Leica) using a 63× oil immersion objective. Images were obtained then analyzed using the software package ImageJ version 1.34a (National Institutes of Health, Bethesda, MD, USA). Digitally reconstructed images of x-z sections (225 mm × appropriate height) with 30 μm spaced y-series slices were created using the “Reslice” function of ImageJ. Image series of x-z sections were processed using the “Find Edges” function and the peak height was calculated with ImageJ. Each analysis was independently repeated three times for each bacterial strain and each bacterial strain was tested in triplicate.

Statistical analysis

Statistical analyzes were conducted using the computational software package GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA). Intergroup differences in each analysis were analyzed using analysis of variance (ANOVA). The Bonferroni correction was used as post-hoc analysis. A P value of less than 0.05 was considered to be statistically significant.

Results

Inhibitory effects of mouth rinse and antimicrobial agents on S. mutans

Growth of all S. mutans strains tested (serotypes c, e, f, and k) was drastically reduced in the presence of ≥ 0.1% mouth rinse compared with growth without the mouth rinse (P < 0.001) (Fig. 1A). Similar results were obtained when chlorhexidine gluconate was used at a concentration equivalent to the amount in 0.1% mouth rinse (Fig. 1B). No significant differences in inhibitory effects were observed among the four S. mutans strains. No inhibitory effects of ethanol or green tea extract were observed with the S. mutans strains except the serotype k strain which was inhibited with a higher concentration of ethanol, equivalent to the amount in 10% mouth rinse (Fig. 1C, D).

Inhibitory effects of mouth rinse and antimicrobial agents on mutants streptococci

Mouth rinse and chlorhexidine gluconate at concentrations ≥0.1% significantly inhibited the growth of S. mutans TW295, a strain isolated from the blood of a subject with bacteremia after tooth extraction, when compared with growth in the absence of either agent (P < 0.001) (Fig. 2A, B). Similar results were observed using a typical S. mutans oral isolate, MT8148, and other cariogenic mutants streptococci (S. sobrinus, serotypes d and g). No growth inhibition was observed for TW295, MT8148 or S. sobrinus strains when ethanol or green tea extract were added (Fig. 2C, D).

Inhibitory effects of mouth rinse and antimicrobial agents on other oral streptococci

The inhibitory effects of the mouth rinse and individual antimicrobial components on other streptococci such as S. oralis, S. gordonii, S. mitis, and S. salivarius, all of which are commensal, non-cariogenic oral bacteria, were analyzed. All of these strains except S. gordonii were inhibited by the mouth rinse or chlorhexidine gluconate at concentrations ≥1%, an effective concentration 10-fold higher than observed for cariogenic mutants streptococci (Fig. 3A, B). Among this group of isolates, S. gordonii growth was inhibited at a concentration of mouth rinse or chlorhexidine gluconate 100-fold higher than the concentration that inhibited mutants streptococcal growth. A concentration of 10% ethanol was able to significantly inhibit growth of S. oralis and S. salivarius compared with growth inhibition of other streptococci tested (P < 0.05 and P < 0.01, respectively) (Fig. 3C). Green tea extract was only able to slightly inhibit the growth of S. salivarius (Fig. 3D).

Inhibitory effects of mouth rinse and antimicrobial agents on P. gingivalis

The antimicrobial effect of the mouth rinse and chlorhexidine gluconate against two P. gingivalis strains that have different virulence in periodon-
among the strains as determined using ANOVA with Bonferroni correction (***, *P* < 0.001).

Significant differences in bacterial growth were observed among the strains as determined using ANOVA with Bonferroni correction (***, *P* < 0.001).

**Fig. 1** Inhibition of growth in *S. mutans* strains cultured with mouth rinse formulated with chlorhexidine gluconate, ethanol, and green tea extract, or with each individual component. *S. mutans* strains were cultured in the presence of (A) the mouth rinse, (B) chlorhexidine gluconate, (C) ethanol, or (D) green tea extract. Values are expressed as the mean ± standard deviation using data from three independent samples. Significant differences in bacterial growth were observed among the strains as determined using ANOVA with Bonferroni correction (***, *P* < 0.001).

**Fig. 2** Inhibition of growth in strains of mutans streptococci cultured with mouth rinse formulated with chlorhexidine gluconate, ethanol, and green tea extract, or with each individual component. Strains of mutans streptococci were cultured in the presence of (A) the mouth rinse, (B) chlorhexidine gluconate, (C) ethanol, or (D) green tea extract. Values are expressed as the mean ± standard deviation using data from three independent samples. Significant differences in bacterial growth were observed among the strains as determined using ANOVA with Bonferroni correction (***, *P* < 0.001).

**Inhibitory effects of mouth rinse and chlorhexidine gluconate on biofilms formed by *S. mutans***

In confocal scanning laser microscopic images, biofilms formed by *S. mutans* MT8148 were drastically reduced in the presence of mouth rinse even at very low concentrations (Fig. 5A). The quantity of formed biofilms was reduced in a dose-dependent manner when mouth rinse was added; the inhibitory effect on both strains was similar when chlorhexidine gluconate was used. High concentrations of ethanol or green tea extract were able to inhibit growth of these strains; however, the inhibition was not as pronounced as the inhibition observed with mouth rinse or chlorhexidine gluconate (Fig. 4C, D).

**Inhibitory effects of mouth rinse and chlorhexidine gluconate on biofilms formed by *P. gingivalis***

Inhibitory effects of mouth rinse and chlorhexidine gluconate were much higher than the concentration necessary for growth inhibition with mouth rinse (Fig. 5D). These results indicate the most effective antimicrobial component in ConCoolF is chlorhexidine gluconate. ConCoolF is recommended to be used by dilution with water from 1% to 0.01%

**Discussion**

Using molecular microbiology techniques, a large number of novel genes or genotypes have been identified in oral bacteria that are closely related to the ability of the strain to cause virulence in oral and systemic diseases [14,24]. Therefore, it may be important to demonstrate that antimicrobial agents are effective against oral bacteria with different characteristics to allow for increased efficacy in patients with a wide variety of oral bacterial species in their oral cavity. In the present study, the antimicrobial effects of a mouth rinse containing a mixture of chlorhexidine gluconate, ethanol, and green tea extract, using a variety of oral bacterial strains were evaluated.

Chlorhexidine gluconate, ethanol, and green tea extract have been widely used as antimicrobial agents [2,3]. A mouth rinse (ConCoolF) formulated with these three agents is commercially available in Japan, in which ethanol and green tea extracts are also used as solubilizing and flavoring agents, respectively. In the present study, low concentrations of ConCoolF or chlorhexidine gluconate effectively inhibited bacterial growth in most of the strains tested. The inhibitory effects of ethanol and green tea extract were quite low, despite their high concentrations in ConCoolF relative to chlorhexidine gluconate (ethanol: 14.7%, green tea extract: 0.40%, chlorhexidine gluconate: 0.05%). These results indicate the most effective antimicrobial component in ConCoolF is chlorhexidine gluconate.

ConCoolF is recommended to be used by dilution with water from 0.1% to 1%. In this study, the inhibitory effects of ConCoolF and its individual antimicrobial components from a concentration of 0.001% were examined, which is lower than that used as mouth wash. In addition, ConCoolF, chlorhexidine, and green tea extracts with concentrations...
higher than 10% are strongly colored, which makes it difficult to accurately measure the absorbance. Therefore, experiments were conducted using the antimicrobial components in concentrations less than 10%.

In the present study, the inhibitory effects of the antimicrobial agents in ConCoolF using S. mutans strains isolated from Japan and Thailand were analyzed. There was no difference observed in the concentration of each antimicrobial agent necessary to inhibit bacterial growth in the S. mutans strains isolated from Japanese or Thai subjects. This result indicates that chlorhexidine gluconate alone or the mixture of the agents (ConCoolF) may be effective on most S. mutans strains regardless of the country they are isolated from, although S. mutans strains isolated from only two countries were tested. Further studies are needed to evaluate the inhibitory effects of these agents on S. mutans strains isolated from countries other than Japan and Thailand.

Mutans streptococci, which include strains of S. mutans and S. sobrinus, are considered to be major etiological agents of dental caries [5]. S. mutans strains are classified into four serotypes (c, e, f, and k) [6] and S. sobrinus are classified into two serotypes (d and g) [7]. In the present study, the effects of ConCoolF and the individual components on mutans streptococci of each serotype isolated from humans were evaluated. These assays revealed that low concentrations of chlorhexidine gluconate or ConCoolF were effective for all serotypes of mutans streptococci that are carried by humans, whereas ethanol and green tea extract had no effect on bacterial growth. Chlorhexidine gluconate or ConCoolF can be efficacious in individuals with S. mutans strains of different genotypes in their oral cavity. Such patients are reported to have higher dental caries activity compared with individuals hosting a single strain [25].

Antimicrobial effects of both mouth rinse and chlorhexidine gluconate were observed in S. mutans strains isolated either from oral cavities or from blood of a patient with bacteremia. S. mutans strain TW295, isolated from blood of a patient with bacteremia, is classified into two serotypes (c and e) [27]. Thus, mouth rinse may be useful for the prevention of systemic diseases induced by S. mutans.

In the present study, the inhibitory effects of chlorhexidine gluconate, ethanol, green tea extract, and the mixture of these agents (ConCoolF) on the growth of various oral streptococci, including non-mutans streptococci were compared. These oral streptococci are considered to be commensal non-cariogenic bacteria that are harmless to healthy human hosts [10,11]. The inhibitory effect of chlorhexidine gluconate or the mouth rinse mixture on bacterial growth in these oral streptococci was approximately 1-100-fold lower than that observed for mutans streptococcal strains. The results indicate an advantage for the application of chlorhexidine gluconate or ConCoolF as they may be effective against cariogenic bacteria without disrupting the normal oral flora.

Both chlorhexidine and some antibiotics such as β-lactam, quinolones, and aminoglycosides are known to damage the cell membranes of bacteria [27]. Thus, bacteria that are less sensitive to chlorhexidine also have low susceptibility to these antibiotics [27]. In the present study, S. oralis, S. gordonii, and S. mitis, all of which are classified as mitis group streptococci, showed lower susceptibility to chlorhexidine than S. mutans. Since previous studies showed that the mitis group streptococci had low sensitivity to β-lactam antibiotics [28], the low sensitivity of the mitis group streptococci to chlorhexidine is reasonable. In the previous study, differences in cell membrane structure were found between bacteria sensitive and resistant to chlorhexidine. Therefore, it is speculated that differences in the cell membrane structure between S. mutans and mitis group bacteria may cause different susceptibility to chlorhexidine.

This is the first study focusing on the inhibitory effects of mouth rinse and its individual antimicrobial components on P. gingivalis strains with
different genotypes. In the past 20 years, many epidemiological studies and experimental research reports have strongly suggested that oral *P. gingivalis* is closely associated with systemic diseases [29]. *P. gingivalis* strains with the type II FimA are highly virulent for both periodontal and systemic diseases, whereas strains with the type I FimA exhibit low virulence [14]. ConCoolF or chlorhexidine gluconate could be useful for the prevention of periodontitis and possibly systemic diseases induced by *P. gingivalis*. Further studies should be performed focusing on the inhibitory effects of ConCoolF and chlorhexidine gluconate against other periodontopathic bacterial species.

Low concentrations of chlorhexidine penetrate the cell wall and damage the cell membrane, and high concentrations of chlorhexidine rapidly enter bacterial cells to coagulate nucleic acid [30], which may be common effects to *S. mutans* and *P. gingivalis*. However, the concentrations at which ConCoolF and chlorhexidine gluconate had inhibitory effects on bacterial growth or biofilm formation were different between *S. mutans* and *P. gingivalis*. This difference may be explained by oxygen, which has been shown to influence the antimicrobial effects induced by some antimicrobial materials [31]. The oxygen requirement may be an important factor in the different inhibitory effects in the present study, since *S. mutans* is a facultative anaerobic bacterium, whereas *P. gingivalis* is an anaerobic bacterium. Future research should analyze in detail the influence of the oxygen requirements of bacteria on the antimicrobial effects of chlorhexidine.

Interestingly, the concentrations at which mouth rinse or chlorhexidine gluconate could inhibit biofilm formation induced by pathogenic bacteria were different, though the inhibitory effects against bacterial growth (non-biofilm) were observed at similar concentrations. Concentrations of 0.01% or higher of both mouth rinse and chlorhexidine gluconate effectively inhibited biofilm formation by *S. mutans*. However, at a concentration less than 0.001%, mouth rinse inhibited biofilm formation in a dose-dependent manner, whereas with concentrations of 0.001% or less of chlorhexidine gluconate no obvious inhibition was observed. These results indicate that components other than chlorhexidine gluconate in mouth rinse may be needed to prevent biofilm formation by *S. mutans*. Unexpectedly, chlorhexidine gluconate had a greater inhibitory effect against biofilm formation of *P. gingivalis* compared with mouth rinse. The effect of chlorhexidine gluconate on *P. gingivalis* biofilm formation may be inhibited by the ethanol and green tea extract in the mouth rinse, in contrast to the results obtained for *S. mutans*.

Although the concentration of chlorhexidine needed to inhibit bacterial growth of *P. gingivalis* was the almost same as that of ConCoolF, the concentrations required to inhibit biofilm formation were different between ConCoolF and chlorhexidine. These results may be a result of the fact that the mechanisms of growth and biofilm formation are different. Bacterial growth is mainly caused by sugar metabolism [32], whereas biofilm formation induced by *P. gingivalis* is thought to involve a cell-surface fimbrillin protein (FimA) and ginguipain [33]. Accordingly, the inhibitory mechanisms of ConCoolF and chlorhexidine may be different between bacterial growth and biofilm formation.

Since chlorhexidine strongly inhibited biofilm formation induced by *P. gingivalis* as compared to ConCoolF, ethanol or green tea extract may reduce the effect of chlorhexidine. In fact, the inhibitory effects of some
antimicrobial materials have been shown to be influenced by ethanol and green tea [34,35]. In addition, the effect of chlorhexidine was reported to be influenced by ethanol [36], although the mechanisms remain unknown. Further studies should be performed focusing on the inhibitory mechanism of the antibiotic agents in mouth rinse against biofilm formation by pathogenic bacteria.

In the present study, ConCoolF and chlorhexidine gluconate could inhibit saliva-coated biofilm formation induced by S. mutans and P. gingivalis. The inhibitory effects may be influenced by the characteristics and composition of saliva. Saliva contains important antimicrobial components such as lysozyme and lactoferrin [37]. Therefore, further research to clarify the precise inhibition mechanism should be performed, focusing on the interaction between salivary components and ConCoolF and its components.

In summary, mouth rinse formulated with chlorhexidine gluconate, ethanol, and green tea extract could inhibit the growth of multiple oral bacterial species, especially cariogenic bacterial species. Among the components of the mouth rinse, chlorhexidine gluconate was the most important for the inhibition of bacterial growth. In addition, mouth rinse or chlorhexidine gluconate could reduce biofilm formation by both S. mutans and P. gingivalis, although the concentrations needed to inhibit biofilm formation were different between the mouth rinse and chlorhexidine gluconate, depending on the bacterial species. These results suggest that using a mouth rinse that contains chlorhexidine gluconate may be a useful method for reducing pathogenic oral bacterial species.

Acknowledgments

The authors declare that they have no conflict of interest.

Conflict of interest

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