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Original

Microbiological assessment of effects of clinical mouth rinses on common oral microbes

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Abstract: Dry mouth occurs frequently in aged individuals, as well as in patients who are hospitalized, receiving multiple drugs, undergoing radiation treatment to the head and neck, or wearing a removable denture prosthesis, use of mouth rinse being often an option for relief. In the present study, we performed microbiological assessments of subjects given three different commercially available mouth rinses commonly employed in clinical practice (Peptisal, Biotène, ConCool) to determine their effects. For bacterial clearance *in vitro*, Peptisal showed the highest level of suppression of oral indigenous bacteria found in both planktonic formations and biofilm. Furthermore, the inhibitory effects of these agents on biofilm formation on acrylic resin plates were examined using scanning electron microscopy. Again, Peptisal proved superior, because acquisition of resistance to antimicrobial peptides by a sensitive microbial strain was rarely observed. We conclude that Peptisal is an effective mouth rinse for clearance of planktonic and biofilm microorganisms present in the oral cavity.

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Introduction

Dry mouth occurs frequently in aged individuals, as well as in patients who are hospitalized, receiving multiple drugs, undergoing radiation treatment to the head and neck, or wearing a removable denture prosthesis. This complication leads to a wide range of minor problems, such as a sore throat, burning sensation, difficulty with speaking and swallowing, hoarseness, and dry nasal passages, as well as more serious issues including poor nutrition, dental problems, and damage to psychological health. Dry mouth is also known to increase the risk of opportunistic infection because of impairment of the antibacterial properties of saliva (1).

Mouth rinsing is often used for relieving the symptoms of dry mouth, and several commercially available products are frequently used in daily and clinical practice, in the context of either self-care or oral care provided by caregivers. Antimicrobial mouth rinses have a variety of clinical applications based on their ability to control the viability and pathogenicity of oral microorganisms. However, there is limited information on the bactericidal ability of various agents (2). In the present study, we conducted microbiological assessments of three different commercially available mouth rinses commonly used in clinical practice.

Materials and Methods

Bacterial strains and conditions

We used a set of orally indigenous bacterial strains kindly provided by Dr. Sumitomo T and Dr. Yamaguchi M (Department of Oral and Molecular Microbiology, Osaka University Graduate School of Dentistry). The tested strains included *Streptococcus mutans* (MT8148), *S. mitis* (ATCC6249), *S. oralis* (ATCC9811), and *S. sanguinis* (ATCC10556), each being an early colonizer of dental biofilm (3), and pneumonia-associated pathogens including *S. pneumoniae* (TIGR4), *S. pyogenes* (MGAS5448), *Candida albicans* (ATCC18804), *Staphylococcus aureus* (NILS1; MRSA, NILS6; MSSA), *Pseudomonas aeruginosa* (ATCC10145), *Serratia marcescens* (ATCC13859), *Klebsiella pneumoniae* (IID5209), and *Escherichia coli* (BL21). The *K. pneumoniae* strain was provided by the Pathogenic Microbes Repository Unit, International Research Center for Infectious Diseases, Institute of Medical Science, the University of Tokyo. *S. mutans*, *S. mitis*, *S. oralis*, and *S. sanguinis* were cultured in Todd-Hewitt broth supplemented with 0.2% yeast extract. *S. pneumoniae*, *S. pyogenes*, *C. albicans*, and *S. aureus* were grown in brain heart infusion broth, and *P. aeruginosa*, *S. marcescens*, *K. pneumoniae*, and *E. coli* in Luria-Bertani broth throughout the experiments.

Ingredients of the tested mouthrinses

Peptisal Gentle Mouthwash: purified water, xylitol, polyglycitol, propylene glycol, hydroxyethyl cellulose, aloe vera juice, natural peppermint oil, poloxamer 407, sodium lactate, citric acid, sodium citrate, macadamia nut oil, polylysine, nisin, lactoferrin; Biotène Dry Mouth Oral Rinse: glycerine, water, sorbitol, xylitol, acrylic acid, hydroxyethyl cellulose, sodium hydroxide; ConCool Mouth rinse: propylene glycol, sorbitol, xylitol, aspartame, acesulfame potassium, sucralose, glycerine, sodium polyacrylate, acrylic acid, PEG-50 hydrogenated castor oil isostearate, PEG-75 lanolin, sodium lauroyl aspartate, fragrance (peppermint), menthol, whey protein milk, ammonium glycyrrhizinate, cetylpyridinium chloride, lactoferrin, aloe vera juice, dextrin, mannitol, human oligopeptide-1.

Analysis of bacterial growth

Overnight cultures were seeded into appropriate medium, and incubated for 24 h at 37°C under aerobic or anaerobic conditions. We tested the effects of the three liquid-type mouth rinses listed above: Peptisal Gentle Mouthwash (T&K Corp., Tokyo, Japan), Biotène Dry Mouth Oral Rinse (GlaxoSmithKline PLC, London, UK), and ConCool Mouth rinse (Weltec Corp., Osaka, Japan), all of which are commercially available in Japan, as well as a Peptisal preparation that did not contain antimicrobial peptides (AMPs). The growth of oral microorganisms was evaluated in medium to which one of the mouth rinses had been added (medium and mouth rinse = 1:1) by measuring the absorbance at 600 nm.

Analysis of biofilm formation

The ability of the tested strains to form biofilms was examined using crystal violet staining of adherent biofilm, as described previously (4,5) with slight modifications. Briefly, overnight cultures were grown in appropriate medium. The medium was then diluted 10-fold with 1% sucrose and culturing was performed in 96-well microtiter plates, each strain being seeded into four wells and incubated at 37°C for 24 h. Preformed biofilms were allowed to develop for 24 h before changing the medium to one containing one of the mouth rinses (medium and mouth rinse = 1:1), and then incubation was continued for another 24 h. After removal of the medium, the plates were washed 3 times with phosphate-buffered saline (PBS), then each biofilm was stained with 0.2% crystal violet for 2 min and washed 3 times with PBS. Stained biofilms were eluted with 100 μL of 100% ethanol and the density of crystal violet staining was determined based on the amount of absorbance at 550 nm (A_{550}).

Observations by scanning electron microscopy

Scanning electron microscope (SEM; JSM-6390JVZ, JEOL Ltd., Tokyo, Japan) observations were performed as described elsewhere (6). Each strain was grown in appropriate medium with 1% sucrose in 24-well plates containing resin (Palapress vario, Heraeus Kulzer GmbH, Hanau, Germany), with the three different mouse rinses individually added and incubated for 24 h at 37°C. Next, the cells were fixed for 1 h with 2% glutaraldehyde at room temperature, then the plates were rinsed 3 times with purified water and the samples dehydrated through a graded series of butanol. Prior to SEM observation, each sample was critical-point dried and sputter-coated with platinum.

Statistical analysis

Data analysis was conducted using SPSS version 20.0 for Windows (SPSS Inc., Chicago, IL, USA). As all the data obtained showed a normal distribution, statistical analysis was performed using one-way analysis of variance (ANOVA) with Tukey's post-hoc analysis. Statistical significance was considered for P values of less than 0.05.

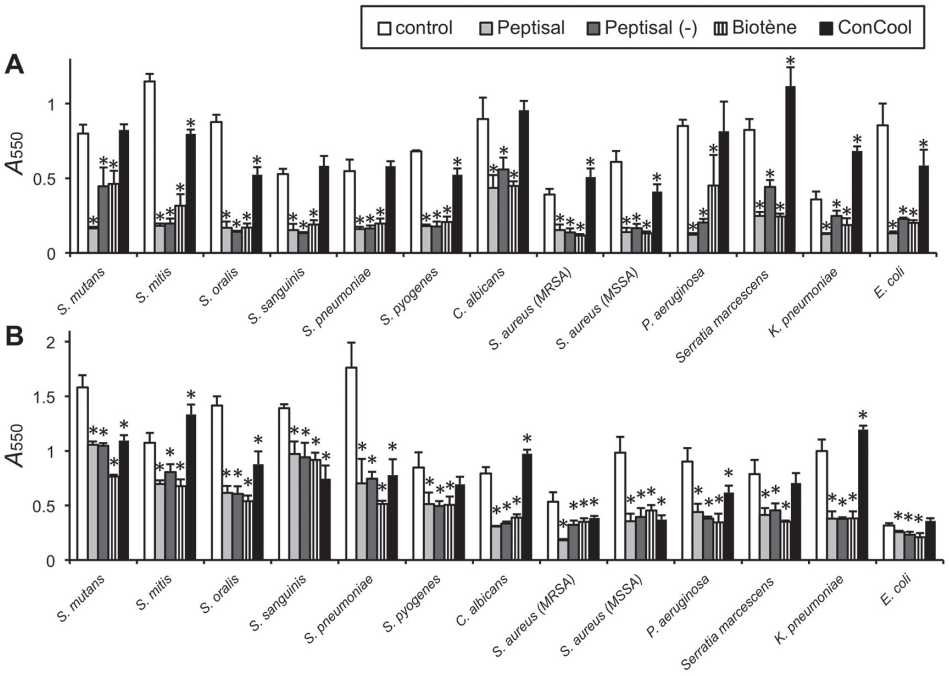

Fig. 1 Analysis of the inhibitory effects of selected mouth rinses on biofilm formation. Each of the tested bacterial strains was inoculated into medium with 1% sucrose for biofilm formation. (A) The examined mouth rinses [Peptisal, Peptisal without AMPs; Peptisal (-), Biotène, ConCool] were added individually. The plates were then incubated at 37°C for 24 h and biofilm was allowed to develop. (B) Biofilms were formed in the absence of any mouth rinse for 24 h, then each mouth rinse was added individually and incubation was continued for another 24 h. Adherent biofilm was stained with crystal violet solution. After elution of the stain with ethanol, the biofilms were quantified by determining the absorbance at 550 nm. All experiments were performed in quadruplicate with three technical repeats. Statistical significance was determined by one-way analysis of variance (ANOVA), followed by Tukey's post-hoc analysis ($P < 0.05$; vs. control).

Results

First, we assessed planktonic growth inhibition for 24 h by each of the three mouth rinses. Bacterial growth of each of the 13 tested strains was inhibited to the greatest degree by Peptisal, whereas inhibition of MSSA by Biotène, and of *C. albicans*, *K. pneumoniae*, and *E. coli* by ConCool was limited (data not shown).

Next, we evaluated the inhibitory effects of the three mouth rinses on biofilm formation over a 24-h period. Both Peptisal and Biotène showed inhibitory effects against biofilm formation by all of the tested strains (Fig. 1A), while only ConCool demonstrated inhibition of biofilm formation by *S. mitis*, *S. oralis*, *S. pyogenes*, MSSA, and *E. coli*. On the other hand, promotion of biofilm formation by MRSA, *S. marcescens*, and *K. pneumoniae* was seen in cultures with ConCool. Furthermore, Peptisal with AMPs showed greater inhibition of biofilms formed by *S. mutans*, *P. aeruginosa*, *S. marcescens*, *K. pneumoniae*, and *E. coli* as compared to Peptisal without AMPs, whereas there were no differences between Peptisal with and without AMPs on biofilms produced by the other strains.

We also assessed the inhibitory effects of the mouth rinses on growth of preformed biofilms. Peptisal and Biotène both showed significant inhibitory effects on biofilms preformed by all of the tested bacterial and fungal strains, while ConCool showed a limited effect or significantly promoted the growth of biofilms formed by *S. mitis*, *C. albicans*, and *K. pneumonia* (Fig. 1B).

SEM observations of the surface of biofilms formed on acrylic resin plates showed that Peptisal reduced biofilm formation within 24 h. In contrast, thick biofilms were observed in cultures with Biotène and ConCool (Fig. 2). Furthermore, the inclusion of AMPs reduced the effect of Peptisal on biofilm formation by *S. mutans*.

Discussion

Aspiration pneumonia in elderly individuals is an important issue worldwide. Oral hygiene is well known to play a key role in its occurrence, and therefore oral health maintenance is recognized to be critical, especially in aged and dependent individuals. Dry mouth increases the risk of opportunistic infections such as candidiasis (1). In addition, Murakami et al. have reported that a denture prosthesis can function as a reservoir for *Candida* spp., especially in individuals with dry mouth (7).

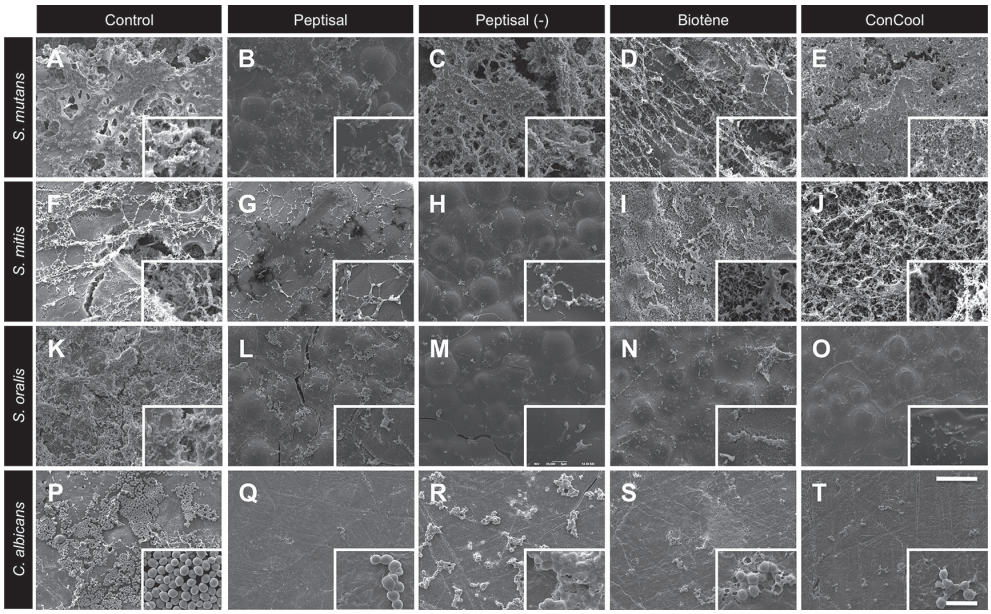

Fig. 2 Scanning electron microscopy observations of biofilms formed on acrylic resin plates. Biofilms formed by *S. mutans* (A-E), *S. mitis* (F-J), *S. oralis* (K-O), and *C. albicans* (P-T) were examined with a scanning electron microscope. Control biofilms (A, F, K, P) were formed over a period of 24 h without added mouth rinse. Other biofilms were formed in the presence of Peptisal (B, G, L, Q), Peptisal without AMPs (C, H, M, R), Biotène (D, I, N, S), or ConCool (E, J, O, T). Biofilms were observed at both lower (white bar in large window: 50 μm) and higher (white bar in small window: 10 μm) levels of magnification.

The present comparisons of bacterial clearance by commercially available mouth rinses showed that Peptisal was most effective for suppression of oral planktonic growth (data not shown) and inhibition of biofilm formation (Fig. 1) by both gram positive and negative bacteria, as well as fungus. Furthermore, the matrix of the product Peptisal, with AMPs removed, was as effective as that including AMPs. Peppermint oil is listed as one of the ingredients of Peptisal and is known to possess a broad spectrum of antimicrobial activities (8). On the other hand, whey protein, a collection of globular proteins included in the list of ingredients of ConCool, might have caused the increased microbial proliferation observed in assays of that product. Lactoferrin, which is known to have antimicrobial activity, is another ingredient of ConCool, although it showed no inhibitory effects on bacterial growth under the current test conditions. Parabens (methyl and propyl 4-hydroxybenzoate), which has a broad spectrum of bacteriostatic abilities, is included as a preservative in Biotène Dry Mouth Oral Rinse, and biofilm formation was potently inhibited by this additive in our assays. Parabens is one of the most commonly used preservatives in topical pharmaceutical preparations, cosmetics, skin care products, and medications, as well as processed foods. However, a widely noted disadvantage of these preservatives is allergic hypersensitivity (contact dermatitis and/or mucositis) (9).

Two AMPs (polylysine and nisin) are included as antimicrobial agents in Peptisal. Of these, polylysine is produced by bacterial strains of the genus *Streptomyces* and used as a food preservative in Japan, Korea, and the United States. It has been shown that ε-polylysine exhibits broad antimicrobial activities against yeast, fungi, and Gram-positive and -negative bacteria (10). Nisin is also a bacteriocin with a broad spectrum of activities against Gram-positive and -negative bacteria, and also used as a food additive (11). We speculated that these AMPs may work to clear planktonic accumulation and biofilm formation by bacteria. Acquisition of resistance to AMPs is quite rare in comparison with conventional antibiotics, in view to the mechanism by which they disrupt bacteria (12).

In the present *in vitro* study, we evaluated the degree of biofilm inhibition by immersion in each of the three tested mouth rinses for 24 h, an assay condition that is not practical for daily usage of oral care products (13). Therefore, further clinical examinations of their antimicrobial effects under rinsing conditions similar to those employed by users of the products, including the period of contact between the mouth rinse and oral microbes, will be needed. In addition, clinical assessments of the products when used in accordance with the manufac-

turers' protocols will be necessary.

A reasonable strategy for efficient control of both dry mouth symptoms and pneumogenic bacteria existing in the oral cavity is considered possible, and usage of a mouth rinse with antimicrobial activity would be beneficial for aged, dependent, and denture-wearing individuals, as well as for caregivers. The present study was limited, as only *in vitro* analyses were performed, and therefore additional examinations will be needed for evaluation of the true clinical effects of these agents. On the basis of our findings, we conclude that Peptisal is an effective mouth rinse with the ability to reduce microorganisms commonly found in both planktonic form and biofilms, as well as on the surfaces of denture materials.

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Conflict of interest

All authors had full access to all of the data obtained, and each takes complete responsibility for the integrity of the data and accuracy of the analysis.

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