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Evaluation of the time-dependent efficacy of commercial denture or orthodontic appliance cleansers: An *in vitro* study

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The aim of this study was to evaluate the antimicrobial, stain and protein removal efficacy of denture or orthodontic appliance cleansers using *in vitro* test methods. Experimental cleansers were applied for experimental time in each evaluation method. To evaluate the microorganism removal efficacy, *C. albicans* and *S. mutans* removal rate was calculated from the specimen surface. Stain and protein removal rate was calculated using the spectrophotometer. Experimental cleansers significantly affected the microorganism removal rate for both *C. albicans* and *S. mutans,* as well as the stain and protein removal rates, at each experimental time ($p<0.05$). As the application time increased, the stain and protein removal rates of all experimental cleansers significantly increased ($p<0.05$). The present study provided *in vitro* evaluation methods to assess the efficacy of denture or orthodontic appliance cleansers. Also, manufacturers and researchers can predict clinical outcomes and ensure proper hygiene management of dentures or orthodontic appliances.

Keywords: Denture cleanser, Cleaning retainer, Antimicrobial, Stain removal, Protein removal

INTRODUCTION

Although the loss rate of all permanent teeth in adults is declining, the number of elderly people requiring dentures continues to increase. In order to maintain a healthy oral environment for patients with dentures, care and maintenance of dentures is essential. The presence of oral biofilm on the denture surface has been associated with denture stomatitis as well as with more serious systemic conditions, especially in elderly patients[1,2]. Poor denture hygiene causes the accumulation of plaque and calculus, bad breath, and aesthetic problems. Plaques on the denture surface are complex aggregates of oral bacteria, fungi, and other organisms, estimated to contain more than 10 organisms per milligram and to involve more than 30 species[3-6]. It has also been determined that dental plaque accumulates more readily on rough denture surfaces than on smooth ones[7,8].

For the removal of food debris and dental plaque formed on the denture surface, mechanical and chemical methods can be considered. Mechanical methods include brushing using a detergent or toothpaste for dentures, and cleaning using an ultrasonic device[9,10]. The agents used in chemical methods include alkaline peroxide, sodium hypochlorite, disinfectants, and enzymes. These chemical methods have been mainly recommended for patients who cannot correctly brush their dentures, such as the elderly or disabled patients, and are reported as an effective method for cleaning areas where the brush cannot reach[11,12].

Denture cleansers that can be easily purchased and used by patients for denture hygiene management are composed of alkaline peroxide. They include powder or tablets that form an alkaline solution of hydrogen peroxide when dissolved in water. These cleansers usually combine alkaline detergents to reduce the surface tension, and either sodium perborate or percarbonate to release oxygen from the solution[13,14]. Oxidizing agents help to remove stains and show some antibacterial action[15]. In addition, some products containing enzymes show very high efficacy when used as an overnight soak to remove heavy denture deposits[15].

The importance of denture hygiene management has been emphasized, and as the number of denture users increases, the market for denture cleanser has grown as well. Among the commercially available denture cleansers sold on the market, many of them are effervescent tablets. The manufacturer demonstrates their antibacterial, antifungal, cleaning, and protein removal effects by labelling them with their efficacy[16-18]. In addition, as the number of patients using removable orthodontic appliances increases, orthodontic appliance cleansers that are marketed for sterilization, cleaning, and protein removal[19-22].

Dentures or orthodontic appliance cleansers have been used by patients for a long time, and many *in vitro* and *in vivo* studies on plaque removal, and the bactericidal and fungicidal effects of cleansers have been conducted. However, since the experimental process, application times, and conditions are different for each study and are not standardized, previous studies have pointed out that it is necessary to develop a reproducible

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standardized method for evaluating the efficacy of denture or orthodontic appliance cleansers[15,21-23]. The American Dental Association (ADA) guide also highlighted the need for further research because there is no standard or guideline to aid evaluation of these cleansers, although cleansers do have to meet requirements pertaining to stain removal, biofilm removal, and antimicrobial effects[1]. Nevertheless, there have been few studies comparing the evaluation of various efficacies of commercially available denture or orthodontic cleansers[23-27]. Among these studies, methods related to reproducible protein removal efficacy have not yet been studied.

Therefore, the purpose of this study is to develop reproducible *in vitro* evaluation methods, and based on this, to evaluate the antibacterial and antifungal efficacy, stain removal efficacy, and protein removal efficacy according to the application time of commercially available denture or orthodontic appliance cleansers.

MATERIALS AND METHODS

Preparation of experimental solution

Seven commercially available denture or orthodontic appliance cleansers were used as experimental cleansers (Table 1). A denture or orthodontic appliance cleanser solution was freshly prepared according to the manufacturer's instructions prior to each test. In addition, sterile distilled water (sterilized distilled water for intermediate and external perfusion, JW Pharmaceutical, Seoul, Korea) was used as a negative control solution and sodium hypochlorite solution (10–15% sodium hypochlorite solution, Sigma-Aldrich, St. Loise, MO, USA) was used as a positive control solution.

Antimicrobial efficacy

1. Culture and growth conditions for microorganisms

To evaluate the antimicrobial efficacy of the experimental solution, *Candida albicans* (*C. albicans*; KCTC No. 7965) and *Streptococcus mutans* (*S. mutans*; KCTC No. 5365) were obtained from the Korean Collection for Type Cultures (KCTC; Jeollabuk-do, Korea). *C. albicans* was cultured in yeast mold broth (YM; Becton Dickinson and Co., Franklin Lakes, NJ, USA) at 37±1°C for 24 h under aerobic conditions. *S. mutans* was cultured in brain heart infusion broth (BHI; Difco, Sparks, MD, USA) at 37±1°C for 48 h under aerobic conditions. Both microbial suspensions were adjusted with each culture solution to establish an optical density (OD) value within the range of 0.4–0.6 at 600 nm using a spectrophotometer (ELISA reader, Epoch, BioTek, Winooski, VT, USA).

Table 1 Information on commercially available denture or orthodontic appliance cleanser products used in the present study

Product	Code	Composition
Gold clinic dent tablets (JW Pharmaceutical, Seoul, Korea)	A	Oxon (persulfuric acid compound), sodium perborate monohydrate, everase, sodium bicarbonate, sodium lauryl sulfate, stearic acid magnesium, citric acid, D-sorbitol
Cleadent (Dong-A pharm, Seoul, Korea)	B	Oxon (persulfuric acid compound), sodium perborate, everase, sodium lauryl sulfate, microcrystalline cellulose, sorbitol, magnesium stearate, citric acid, xylitol, sodium bicarbonate
Toughdent (Kobayashi, Osaka, Japan)	C	Oxon (persulfuric acid compound), sodium perborate monohydrate, everase, disodium pyrophosphate, sodium lauryl sulfate, malic acid, citric acid (anhydrous), meta aluminum magnesium silicate, benzotriazole, sorbitol, magnesium stearate, alpha olefin sulfonic acid, sodium carbonate, sodium bicarbonate, polyethylene glycol 6000
Polident (Stafford-Miller, Waterford, Ireland)	D	Oxon (persulfuric acid compound), sodium percarbonate, tetraacetylethylenediamine, sodium benzoate, sodium lauryl sulfate, citric acid (anhydrous), sodium carbonate (anhydrous), VP/VA copolymer, sodium bicarbonate, polyethylene glycol
Clinpro denture cleaner (3M, St. Paul, MN, USA)	E	Oxon (persulfuric acid compound), sodium perborate monohydrate, everase, D-sorbitol, magnesium stearate acid, sodium bicarbonate, hydroxypropyl methylcellulose
Pestseven 8090 Clean dent (Pestseven, Gyeonggi-do, Korea)	F	Oxon (persulfuric acid compound), sodium bicarbonate, sodium perborate monohydrate, citric acid, D-sorbitol, sodium lauryl sulfate, hypromellose, magnesium stearate
Dentfix-forte (Helago-Pharm, Neuhofer Weiche, Germany)	G	Oxon (persulfuric acid compound), sodium bicarbonate, sodium carbonate, citric acid, D-sorbitol, PVP/VA copolymer, sodium lauryl sulfate, sodium lauryl sulfoacetate

2. Evaluation of the antimicrobial effect
To evaluate the antimicrobial effect of the experimental solution, 1 mL of microbial suspension (OD value within the range of 0.4–0.6 at 600 nm) was diluted with 9 mL of the freshly prepared experimental solution and incubated at 37±1°C for 10 min, 30 min, 3 h, 6 h, and 12 h. At this time, 1 mL of microbial suspension was also incubated with 9 mL of each microbial culture medium. After each incubation time, 100 μL of the microbial suspension was spread onto YM (*C. albicans*) or BHI (*S. mutans*) agar plates and incubated at 37±1°C for 48 h before counting the number of viable colony forming units (CFUs) of *C. albicans* and *S. mutans*. The antimicrobial effect of the experimental solution was estimated using the following equation: CFUs reduction rate (%)=(1−(CFUs of microorganism treated with experimental solution/CFUs of microorganisms cultured in microbial culture media))×100.

3. Sample preparation for the microorganism removal test
A thermoplastic orthodontic sheet (Easy-VAC Gasket splint, 3A MEDES, Gyeonggi-do, Korea) with 1.0 mm thickness was selected to prepare the specimen to be exposed to each type of microorganism. The thermoplastic orthodontic sheet was cut to 20×20×1 mm, and one side was polished using an automatic grinder (Buehler, Lake Bluff, IL, USA) with #400 grit silicon carbide paper (Deerfos, Incheon, Korea) under a continuous water stream. The polished specimen was sonicated in distilled water for 5 min to remove any foreign substances remaining on the sheet surface, air-dried, and then sterilized using ethylene oxide gas. The sterilized specimen was placed on a 6-well cell culture plate (SPL Life Science, Gyeonggi-do, Korea) and 60 μL of microbial suspension was spread onto the surface, which was subsequently covered with a piece of sterilized polyethylene film measuring 17×17 mm. The film was gently pressed down so that the microbial suspension spread to, but did not leak beyond, the edges of the film. The plate was incubated at 37±1°C with a relative humidity of not less than 90% for 24 h. After carefully removing the polyethylene film, the specimen exposed to each type of microorganism was positioned in the 6-well cell culture plate facing up. Two specimens were selected to confirm the attachment of microorganisms on the surface. The specimens were then fixed with Karnovsky's fixative solution (2% paraformaldehyde; 2% glutaraldehyde; 0.1 M phosphate buffer) for 24 h, washed with 0.1 M phosphate buffer for 30 min, and post-fixed with OsO_4 for 2 h. The specimen was then dehydrated with a series of graded ethanol solutions (50–100%) and dried using the critical point dryer (Leica EM CPD300, Leica, Wien, Austria) for 2 h. The specimens were then sputter-coated with platinum using an ion sputter (Leica EM ACE600, Leica) and observed with a field emission scanning electron microscope (FE-SEM; Merlin, Carl Zeiss, Oberkochen, Germany) with an accelerating voltage of 15.00 kV.

4. Evaluation of microorganism removal efficacy
After carefully removing the polyethylene film, the specimen attached to each microorganism was positioned in a 6-well cell culture plate facing up. A micropipette tip was placed on the wall of the plate, and 3 mL of freshly prepared experimental solution was dispensed. After confirming that the specimen was completely immersed in the experimental solution, it was stored at 25±2°C for 10 min, 30 min, 3 h, 6 h, and 12 h. When applying the experimental solution, the micropipette tip was not in direct contact with the specimen surface. The specimen was washed by gentle shaking in a beaker containing phosphate buffered saline (PBS; Welgene, Gyeongsangbuk-do, Korea). The washing process was repeated twice so that the experimental solution did not remain on the specimen surface. The specimen was immersed in 1 mL of each microbial culture medium and sonicated for 10 min to detach the microbes from the specimen surface. At this time, the specimen not to be immersed in the experimental solution was also prepared using the same microbial collection method. After collecting the microorganism from the specimen, the obtained microbial suspension was diluted, applied onto the YM (*C. albicans*) or BHI (*S. mutans*) agar plates, and incubated at 37±1°C for 48 h before counting the number of viable CFUs of *C. albicans* and *S. mutans*. To evaluate the microorganism removal efficacy of the experimental solution, the microorganism removal rate was calculated with CFUs using the following equation: Microorganism removal rate (%)=(1−(CFUs of microorganisms remaining on the specimen surface after treatment with experimental solution/CFUs of attached microorganism on the specimen surface))×100.

Stain removal efficacy
1. Preparation for the stain removal test
The artificial saliva used as stain solution was prepared using the following formula: 0.4 g NaCl, 0.4 g KCl, 0.78 g $NaH_2PO_4 \cdot 2H_2O$, 0.005 g $Na_2S \cdot 9H_2O$, 1 g urea, 0.795 g $CaCl_2 \cdot 2H_2O$, 1 L distilled water, and 1.4 g of type II mucin (Sigma-Aldrich). To make homogeneous artificial saliva, the mixture was stirred with an agitator for 1 h and sterilized at 121°C for 15 min using the autoclave (SJP-40AC, Sejong scientific, Gyeonggi-do, Korea). A 96-well cell culture plate (SPL Life Science) was used as the stain surface. Furthermore, 100 μL of sterilized artificial saliva was dispensed into each well and stored in the drying oven (TD039, Daihan Scientific, Gangwon-do, Korea) at 50±2°C for 12 h. When the saliva was completely dried from each well, after cooling down at 25±2°C, the absorbance was measured at 600 nm using an ELISA reader. This procedure was repeated until an OD value of 0.4–0.6 was recorded on the plate.

2. Evaluation of stain removal efficacy
The baseline OD value of the stained plate was measured, and a column of eight rows in the 96-well cell culture plate was allocated for each experimental solution. A micropipette tip was placed on the wall of the 96-well cell culture plate, and 100 μL of the freshly prepared

experimental solution was dispensed. When applying the experimental solution, the micropipette tip was not in direct contact with the stained plate surface. After confirming that the stained well plate was completely filled with the experimental solution, it was stored at 25±2°C for 10 min, 30 min, 3 h, 6 h, and 12 h. Each well was then rinsed with 100 μL of distilled water. The rinsing process was repeated twice so that the experimental solution did not remain in each well. The rinsed well plate was stored at 50±2°C for 20 min to dry and kept in a desiccator at 25±2°C until used. The OD value was then measured at 600 nm using an ELISA reader. To evaluate the stain removal efficacy of the experimental solution, the stain removal rate was calculated using the following equation: Stain removal rate (%)=(1−(OD_{600} of well plate treated with experimental solution/OD_{600} of well plate covered with dried artificial saliva))×100.

Protein removal efficacy

1. Sample preparation for the protein removal test

A thermoplastic orthodontic sheet (Easy-VAC Gasket splint, 3A MEDES) with 1.0 mm thickness was selected to prepare the specimen coated with bovine serum albumin (BSA). The sheet was cut to 10×10×1 mm. A piece of sheet was placed on a glass Petri dish and 50 μL of BSA solution (2,000 μg/mL, Thermo Scientific, Rockford, IL, USA) was dropped onto one side. The BSA solution was carefully spread through the tip of the micropipette so that the specimen surface was completely covered with the BSA solution. At this time, 50 μL of BSA solution was maintained on the specimen surface, and care was taken not to spill the solution on the glass Petri dish. The glass Petri dish with the specimen covered with BSA solution was allowed to dry at 50±2°C for 2 h. The specimen surface was visually checked to be covered with dried BSA and kept in a desiccator at 25±2°C to cool down at 25±2°C.

2. Evaluation of protein removal efficacy

The specimen covered with dried BSA was placed on a 12-well cell culture plate (SPL Life Science) facing up. A micropipette tip was placed on the wall of the plate and 2 mL of the freshly prepared experimental solution was dispensed. After confirming that the specimen was completely immersed in the experimental solution, it was stored at 25±2°C for 10 min, 30 min, 3 h, 6 h, and 12 h. When applying the experimental solution, the micropipette tip was not in direct contact with the specimen surface. The specimen applied with the experimental solution was washed by gentle shaking in a beaker containing distilled water. The washing process was repeated twice so that the experimental solution does not remain on the specimen surface. The specimen was stored at 50±2°C for 20 min to dry and kept in a desiccator at 25±2°C until further measurement. At this time, the specimen covered with dried BSA to not be immersed in the experimental solution was also prepared. To make the bicinchoninic acid (BCA) working reagent, 50 mL of reagent A (Pierce™ BCA protein assay reagent A, Thermo Fisher Scientific), and 1 mL of reagent B (Pierce™ BCA protein assay reagent B, Thermo Fisher Scientific) were mixed using a vortex device (Vortex Mixer VM1, LABTron, Seoul, Korea) for 10 s at 25±2°C. A dried specimen was placed on a 12-well cell culture plate, and 50 μL of the prepared BCA working reagent was dropped onto the specimen surface. The BCA working reagent was carefully spread through the tip of the micropipette so that the specimen surface was completely covered. At this time, 50 μL of BCA working reagent was maintained on the specimen surface, and care was taken not to spill the solution on the plate. The culture plate was then stored at 37±1°C for 30 min. BCA working reagent was then applied to the specimen surface covered with dried BSA not to be immersed in the experimental solution. Fifty microliters of reacted BCA working reagent from the specimen surface was collected with a micropipette and dispensed into a 96-well cell culture plate (SPL Life Science). The absorbance of the reacted BCA working reagent was measured at 562 nm using an ELISA reader within 10 min. To evaluate the protein removal efficacy of the experimental solution, the protein removal rate was calculated with the OD value using the following equation: Protein removal rate (%)=(1−(OD_{562} of reacted BCA working reagent on the sheet surface treated with experimental solution/OD_{562} of the reacted BCA working reagent on the sheet surface covered with dried BSA))×100. In addition, a standard curve with known protein concentrations (25, 125, 250, 500, 750, 1,000, 1,500, and 2,000 μg/mL) was drawn using BSA solution to calculate the content of protein remaining on the sheet surface.

Statistical analysis

The antimicrobial, stain removal, and protein removal effects of experimental solutions at the same application time were analyzed using one-way ANOVA (SPSS 25, IBM, NY, USA), followed by Tukey's statistical test. In addition, the antimicrobial, stain removal, and protein removal effects at different application times within the same experimental solution were confirmed using the same method at a significance level of 0.05.

RESULTS

Antimicrobial efficacy

1. CFU reduction rate

Table 2 shows that all denture or orthodontic appliance cleansers significantly affected the CFU reduction rate for both *C. albicans* and *S. mutans* at each application time, when compared with the negative control ($p<0.05$). When denture or orthodontic appliance cleansers were applied to *C. albicans* for 10 min, there was a significant difference in CFU reduction rates between products. However, when denture or orthodontic appliance cleansers were applied to *C. albicans* for 30 min, 3 h, 6 h, and 12 h, all products showed a CFU reduction rate close to 100%, which was not significantly different from the positive control ($p>0.05$). The negative control showed a significantly lower CFU reduction rate at all application times compared to the denture or orthodontic appliance

Table 2 CFU reduction rate (%) of denture or orthodontic appliance cleansers with *C. albicans* and *S. mutans* at five different application times. Each value is the mean±standard deviation for 6 test repetitions

		10 min	30 min	3 h	6 h	12 h
Candida albicans	A	99.1±0.1^{Abc}	99.8±0.0^{Bb}	100.0± 0.0^{Cb}	100.0±0.0^{Cb}	100.0±0.0^{Cb}
	B	99.2±0.5^{Ab}	98.9±0.1^{Bb}	100.0±0.0^{Cb}	100.0±0.0^{Cb}	100.0±0.0^{Cb}
	C	99.7±0.1^{Abc}	99.9±0.1^{Bb}	100.0±0.0^{Cb}	100.0±0.0^{Cb}	100.0±0.0^{Cb}
	D	100.0±0.0^{Ac}	100.0±0.0^{Ab}	100.0±0.0^{Ab}	100.0±0.0^{Ab}	100.0±0.0^{Ab}
	E	100.0±0.0^{Ac}	100.0±0.0^{Bb}	100.0±0.0^{Bb}	100.0±0.0^{Bb}	100.0±0.0^{Bb}
	F	99.9±0.0^{Ac}	100.0±0.0^{Bb}	100.0±0.0^{Bb}	100.0±0.0^{Bb}	100.0±0.0^{Bb}
	G	99.8±0.1^{Abc}	100.0±0.0^{Bb}	100.0±0.0^{Bb}	100.0±0.0^{Bb}	100.0±0.0^{Bb}
	PC	100.0±0.0^{Ac}	100.0±0.0^{Ab}	100.0±0.0^{Ab}	100.0±0.0^{Ab}	100.0±0.0^{Ab}
	NC	55.3±3.8^{Ba}	51.6±4.0^{Ba}	50.7±4.6^{Ba}	50.9±3.7^{Ba}	33.2±3.5^{Aa}
Streptococcus mutans	A	100.0±0.0^{Ab}	100.0±0.0^{Ab}	100.0±0.0^{Ab}	100.0±0.0^{Ab}	100.0±0.0^{Ab}
	B	100.0±0.0^{Ab}	100.0±0.0^{Ab}	100.0±0.0^{Ab}	100.0±0.0^{Ab}	100.0±0.0^{Ab}
	C	100.0±0.0^{Ab}	100.0±0.0^{Ab}	100.0±0.0^{Ab}	100.0±0.0^{Ab}	100.0±0.0^{Ab}
	D	100.0±0.0^{Ab}	100.0±0.0^{Ab}	100.0±0.0^{Ab}	100.0±0.0^{Ab}	100.0±0.0^{Ab}
	E	100.0±0.0^{Ab}	100.0±0.0^{Ab}	100.0±0.0^{Ab}	100.0±0.0^{Ab}	100.0±0.0^{Ab}
	F	100.0±0.0^{Ab}	100.0±0.0^{Ab}	100.0±0.0^{Ab}	100.0±0.0^{Ab}	100.0±0.0^{Ab}
	G	100.0±0.0 ^{Ab}	100.0±0.0^{Ab}	100.0±0.0^{Ab}	100.0±0.0^{Ab}	100.0±0.0^{Ab}
	PC	100.0±0.0 ^{Ab}	100.0±0.0^{Ab}	100.0±0.0^{Ab}	100.0±0.0^{Ab}	100.0±0.0^{Ab}
	NC	48.1±7.2^{Ba}	45.4±6.3^{Ba}	44.6±5.6^{Ba}	45.6±4.0^{Ba}	18.3±4.4^{Aa}

CFUs reduction rate (%)=(1−(CFUs of microorganism treated with experimental solution/CFUs of microorganisms cultured in microbial culture media))×100. The same lowercase letters indicate that there were no differences in CFU reduction rate between experimental groups within each application time; the same uppercase letters indicate no differences in CFU reduction rate between application times within each experimental group (p>0.05). PC: Positive control, NC: Negative control

cleansers and the positive control (p<0.05). When the denture or orthodontic appliance cleansers were applied to *S. mutans* for each application time, every cleanser showed a 100% CFU reduction rate, though this was not a significant difference when compared with the positive control (p>0.05). When the negative control was applied to *S. mutans*, the CFU reduction rate was significantly lower than that of the cleansers and of the positive control (p<0.05).

2. SEM observations of microorganisms on the specimen
SEM was used to visually confirm that two types of microorganisms were attached to the specimen surface. Compared to the unpolished specimen, both types of microorganisms were densely adhered to the polished specimen (Fig. 1). *C. albicans* was attached between the curves of the polished surface, and the enlarged image showed cluster cells. *C. albicans* on the surface of the unpolished and polished specimens was commonly oval-shaped, with differences only in density. *S. mutans* was also attached between the curves of the polished surface, and the enlarged image showed aggregated long chains of diplococci. The shape of *S. mutans* on the surface of the polished specimen was similar to that of the unpolished specimen, with only differences in density.

3. Microorganism removal rate
Table 3 shows that all denture or orthodontic appliance cleansers significantly affected the microorganism removal rate for both *C. albicans* and *S. mutans* at each application time, as compared with the negative control (p<0.05). When the cleansers were applied to *C. albicans* for 10 and 30 min, there was a significant difference in *C. albicans* removal rate between products (p<0.05). However, when the cleansers were applied to *C. albicans* for 3, 6, and 12 h, all products showed a *C. albicans* removal rate of close to 100%, which was not a significant difference when compared with the positive control (p>0.05). The negative control showed a significantly lower *C. albicans* removal rate at all application times compared to the denture or orthodontic appliance cleansers and the positive control (p<0.05). There was no significant difference in the *C. albicans* removal rate according to the application time of the negative control (p>0.05). When the denture or orthodontic appliance cleansers were applied to *S. mutans* for each application time, there was no significant difference between the *S. mutans* removal rate of the cleansers and the positive control (p>0.05). When the negative control was applied to *S. mutans*, the *S. mutans* removal rate was significantly lower than that of the all denture or orthodontic appliance cleansers and the positive control (p<0.05).

Stain removal efficacy
Figure 2 shows that all denture or orthodontic appliance cleansers significantly affected the stain removal rate at each application time, as compared with the negative

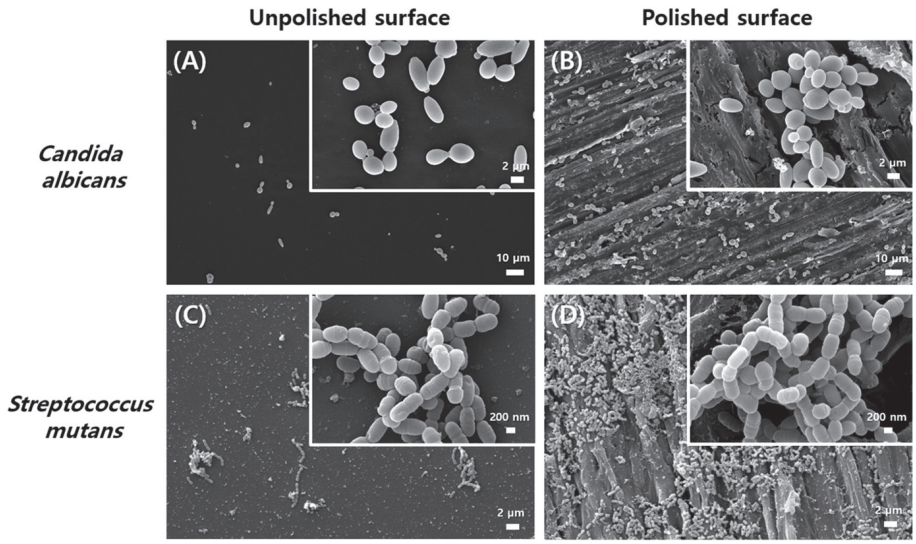

Fig. 1 Scanning electron microscopy images of *Candida albicans* and *Streptococcus mutans* attached on the thermoplastic polymer sheet surface.
Candida albicans attached on the (A) unpolished surface, and (B) polished surface; magnifications: 500× (main image) and 3,000× (inset). *Streptococcus mutans* attached on the (C) unpolished surface, and (D) polished surface; magnifications: 2,000× (main image) and 20,000× (inset).

Table 3 Microorganism removal rate (%) of denture or orthodontic appliance cleansers with *C. albicans* and *S. mutans* at five different application times. Each value is the mean±standard deviation for 6 test repetitions

		10 min	30 min	3 h	6 h	12 h
Candida albicans	A	57.6 ± 5.30^{Ab}	95.9 ± 0.8^{Bbc}	100.0 ± 0.0^{Cb}	100.0 ± 0.0^{Cb}	100.0 ± 0.0^{Cb}
	B	96.3 ± 2.2^{Ad}	95.5 ± 0.5^{Abc}	100.0 ± 0.0^{Bb}	100.0 ± 0.0^{Bb}	100.0 ± 0.0^{Bb}
	C	94.5 ± 1.6^{Ad}	95.4 ± 1.4^{Ab}	100.0 ± 0.0^{Bb}	100.0 ± 0.0^{Bb}	100.0 ± 0.0^{Bb}
	D	98.1 ± 2.7^{Ad}	98.5 ± 0.8^{Bcd}	100.0 ± 0.0^{Bb}	100.0 ± 0.0^{Bb}	100.0 ± 0.0^{Bb}
	E	91.6 ± 4.9^{Acd}	98.3 ± 0.7^{Bbcd}	100.0 ± 0.0^{Bb}	100.0 ± 0.0^{Bb}	100.0 ± 0.0^{Bb}
	F	86.8 ± 4.2^{Ac}	99.5 ± 1.3^{Bd}	100.0 ± 0.0^{Bb}	100.0 ± 0.0^{Bb}	100.0 ± 0.0^{Bb}
	G	98.4 ± 2.0^{Ad}	100.0 ± 0.0^{Bd}	100.0 ± 0.0^{Bb}	100.0 ± 0.0^{Bb}	100.0 ± 0.0^{Bb}
	PC	98.9 ± 1.1^{Ad}	100.0 ± 0.0^{Bd}	100.0 ± 0.0^{Bb}	100.0 ± 0.0^{Bb}	100.0 ± 0.0^{Bb}
	NC	27.1 ± 3.2^{Aa}	28.3 ± 5.6^{Aa}	22.9 ± 6.7^{Aa}	18.5 ± 6.5^{Aa}	20.2 ± 3.04^{Aa}
Streptococcus mutans	A	99.9 ± 0.0^{Ab}	100.0 ± 0.0^{Bb}	100.0 ± 0.0^{Bb}	100.0 ± 0.0^{Bb}	100.0 ± 0.0^{Bb}
	B	99.9 ± 0.1^{Ab}	100.0 ± 0.0^{Bb}	100.0 ± 0.0^{Bb}	100.0 ± 0.0^{Bb}	100.0 ± 0.0^{Bb}
	C	100.0 ± 0.0^{Ab}	100.0 ± 0.0^{Ab}	100.0 ± 0.0^{Ab}	100.0 ± 0.0^{Ab}	100.0 ± 0.0^{Ab}
	D	100.0 ± 0.0^{Ab}	100.0 ± 0.0^{Ab}	100.0 ± 0.0^{Ab}	100.0 ± 0.0^{Ab}	100.0 ± 0.0^{Ab}
	E	100.0 ± 0.0^{Ab}	100.0 ± 0.0^{Ab}	100.0 ± 0.0^{Ab}	100.0 ± 0.0^{Ab}	100.0 ± 0.0^{Ab}
	F	100.0 ± 0.0^{Ab}	100.0 ± 0.0^{Ab}	100.0 ± 0.0^{Ab}	100.0 ± 0.0^{Ab}	100.0 ± 0.0^{Ab}
	G	100.0 ± 0.0^{Ab}	100.0 ± 0.0^{Ab}	100.0 ± 0.0^{Ab}	100.0 ± 0.0^{Ab}	100.0 ± 0.0^{Ab}
	PC	100.0 ± 0.0^{Ab}	100.0 ± 0.0^{Ab}	100.0 ± 0.0^{Ab}	100.0 ± 0.0^{Ab}	100.0 ± 0.0^{Ab}
	NC	48.1 ± 8.3^{Ba}	47.6 ± 7.1^{Ba}	45.2 ± 9.8^{Ba}	45.1 ± 4.5^{Ba}	26.7 ± 9.4^{Aa}

Microorganism removal rate (%)=(1−(CFUs of microorganisms remaining on the specimen surface after treatment with experimental solution/CFUs of attached microorganism on the specimen surface))×100. The same lowercase letters indicate no differences in microorganism removal rate between experimental groups within each application time; the same uppercase letters indicate no differences in microorganism removal rate between application times within each experimental group ($p>0.05$). PC: Positive control, NC: Negative control

control ($p<0.05$). The negative and positive controls showed no significant difference in the stain removal rate between application times ($p>0.05$). However, all denture or orthodontic appliance cleansers showed

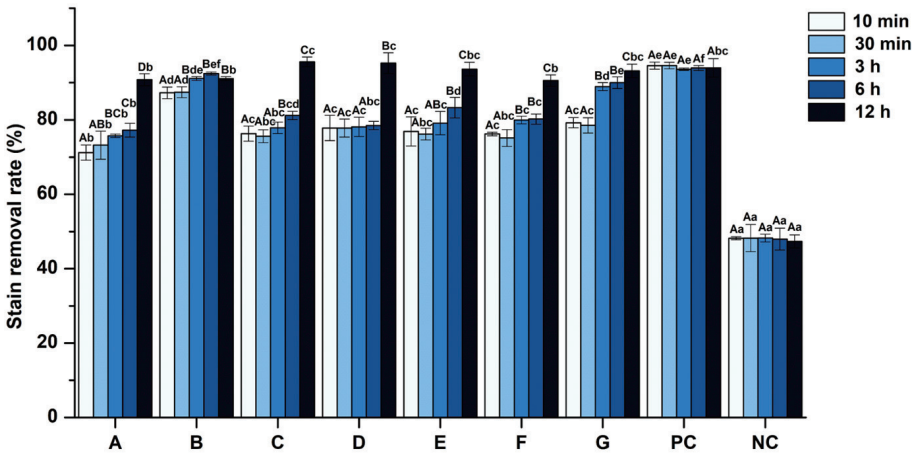

Fig. 2 Stain removal rate (%) of denture or orthodontic appliance cleansers at five different application times.
Each value is the mean±standard deviation for 6 test repetitions. The same lowercase letters mean no differences in stain removal rate between experimental groups within each application time; the same uppercase letters mean no differences in stain removal rate between application times within each experimental group (p>0.05). PC: Positive control, NC: Negative control

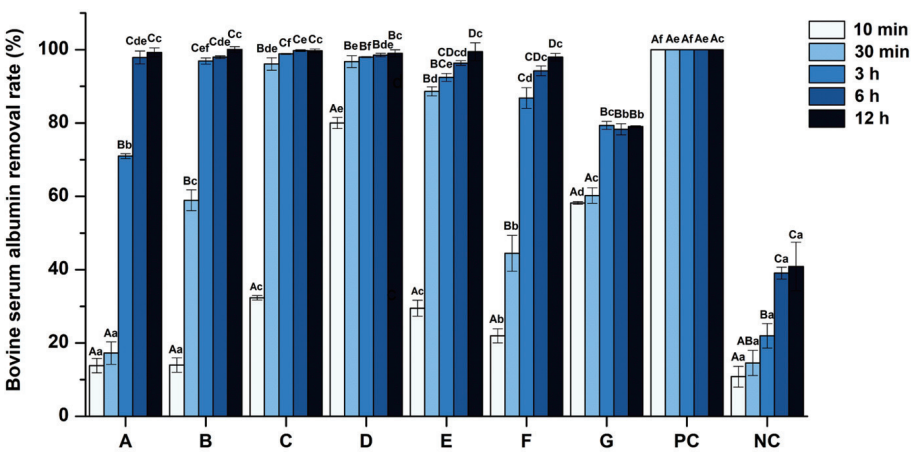

Fig. 3 Protein removal rate (%) of denture or orthodontic appliance cleansers at five different application times.
Each value is the mean±standard deviation for 6 test repetitions. The same lowercase letters mean no differences in protein removal rate between experimental groups within each application time; the same uppercase letters mean no differences in protein removal rate between application times within each experimental group (p>0.05). PC: Positive control, NC: Negative control

significant differences in the stain removal rate between application times (p<0.05). As the application time increased, the stain removal rate of every cleanser significantly increased (p<0.05). In all cleansers, the stain removal rate after a 10 min application did not show a significant difference from the removal rate after a 30 min application (p>0.05). For all cleansers, the stain removal rate calculated after application for 12 h was not significantly different from that of the positive control (p>0.05).

Protein removal efficacy

1. Protein removal rate

Figure 3 shows that all denture or orthodontic appliance cleansers significantly increased the protein removal rate after 30 min as compared to the negative control (p<0.05). The positive control showed no significant difference in protein removal rate between application times (p>0.05); however, the negative control showed significant differences in protein removal rate between application times, and the protein removal rate increased with increasing application time (p<0.05).

Table 4 Remaining protein content (μg/mL) on the specimen after treating with denture or orthodontic appliance cleansers at five different application times. Each value is the mean±standard deviation for 6 test repetitions

Group	10 min	30 min	3 h	6 h	12 h
A	1,820.4±43.0^{Cf}	1,745.9±66.5^{Ce}	574.6±14.1^{Be}	0.0±0.0^{Aa}	0.0±0.0^{Aa}
B	1,816.8±43.0^{Cf}	836.9±61.6^{Bc}	19.1±11.3^{Aab}	0.0±0.0^{Aa}	0.0±0.0^{Aa}
C	1,416.3±13.4^{Cd}	45.7±26.3^{Bab}	0.0±0.0^{Aa}	0.0±0.0^{Aa}	0.0±0.0^{Aa}
D	377.9±33.2^{Bb}	0.0±0.0^{Aa}	0.0±0.0^{Aa}	0.0±0.0^{Aa}	0.0±0.0^{Aa}
E	1,478.4±47.8^{Dd}	190.1±26.8^{Cb}	106.8±23.2^{Bb}	21.8±14.1^{Aab}	0.0±0.0^{Aa}
F	1,643.2±42.2^{De}	1,152.3±106.5^{Cd}	229.1±61.3^{Bc}	67.8±29.3^{Ab}	0.0±0.0^{Aa}
G	852.8±8.1^{Bc}	808.5±46.3^{Bc}	392.1±24.0^{Ad}	415.2±33.2^{Ac}	399.2±5.3^{Ab}
PC	0.0±0.0^{Aa}	0.0±0.0^{Aa}	0.0±0.0^{Aa}	0.0±0.0^{Aa}	0.0±0.0^{Aa}
NC	1,885.9±61.6^{Cf}	1,804.4±74.7^{BCe}	1,643.2±73.1^{Bf}	1,269.3±35.4^{Ad}	1,230.3±144.3^{Ac}

The same lowercase letters indicate that there are no differences in remaining protein content between experimental groups within each application time; the same uppercase letters indicate no differences in remaining protein content between application times within each experimental group (p>0.05). PC: Positive control, NC: Negative control

All denture or orthodontic appliance cleansers showed significant differences in protein removal rate between application times (p<0.05). As the application time increased, the protein removal rate of every cleanser was significantly increased (p<0.05). For all cleansers, protein removal rate after 6 h application did not show a significant difference from the protein removal rate after 12 h application (p>0.05). In addition, when all cleansers except the G group were applied for 12 h, the protein removal rate was not significantly different from that of the positive control (p>0.05).

2. Protein content remaining on the specimen

The amount of protein on the surface of the specimen covered with dried BSA was 2,015.3±32.3 μg/mL. Table 4 shows that every cleanser significantly affected the protein residuals on the specimen compared to the negative control (p<0.05). The positive control showed no significant difference in the remaining protein content between application times (p>0.05). However, the negative control showed significant differences in the remaining protein content between application times, and the remaining protein content decreased with increasing application time (p<0.05). All cleansers showed significant differences in the remaining protein content between application times (p<0.05). As the application time of every cleanser increased, the remaining protein content significantly decreased (p<0.05). Among the cleansers, remaining protein content after 6 h application did not differ significantly from the remaining protein content after 12 h application (p>0.05). In the G group, the smallest amount of protein was detected after 10 min compared with other cleanser groups (p<0.05), and there was no significant difference in the amount of remaining protein within the G group between application times after 3 h (p>0.05). After 12 h, the G group showed the highest protein content compared to other cleanser groups, but it was less than that of the negative control (p<0.05).

DISCUSSION

With the increasing number of users of removable dentures or orthodontic appliances, the market size of products that can be used for the management of appliance hygiene is growing rapidly. Among them, cleansing tablets, which are used as a chemical hygiene tool, are the most recommended products by dental healthcare professionals as they help effectively manage hygiene without damaging the surface of the appliance even after repeated use[16].

Denture or orthodontic appliance cleansers are dental materials used for the hygiene management of removable devices; *in vitro* assessments and clinical studies on the antibacterial and stain removal effects of these cleaning agents have been carried out using non-standard methods, with many conflicting results reported[28-32]. In addition, studies comparing and evaluating the protein removal effect described by the manufacturer *via* reproducible yet objective testing methods are rare. To overcome these problems, standardized control materials and evaluation methods should be considered, and evaluation methods should be closely related to the clinical use of cleansers. Therefore, in this study, we aimed to establish a method for evaluating antimicrobial, stain removal, and protein removal efficacy. Based on this, the effects of commercially available denture or orthodontic appliance cleansers were compared according to different application times.

The tablet-type denture or orthodontic appliance cleansers used in this study were sold on the market by the manufacturer who described the antimicrobial effect, stain removal effect, and protein removal effect as efficacy. It was recommended that the appliance be

immersed in the cleanser for 10 to 30 min, though the application time presented by each manufacturer varied. To evaluate the antimicrobial effect of the cleansers, a reproducible test method should be used that is capable of observing the effect of removing or inhibiting the growth of bacteria remaining on the surface of the denture or orthodontic appliance[1].

Previous studies related to antimicrobial evaluation have shown that the type of plaque grown (*in vitro* vs. *in vivo*), growth site (directly on the prosthesis, in other materials embedded in a denture, *etc.*), and assessment method (plaque indices, CFUs, SEM, clinical evaluation of tissue indices, and others) were all different[15,23]. In addition, international standards for measuring the antibacterial activity of plastics describe the bacteria used in the test, preparation of test specimens, inoculation of test specimens, recovery of bacteria from test specimens, and calculation of the antibacterial activity[33].

In this study, a method for evaluating the antimicrobial efficacy of denture or orthodontic appliance cleansers was established by applying methods from previous studies and international standards ISO 22196:2011[17,33-35]. When the removable device is immersed in the cleanser, the microorganisms are detached from the appliance and float in the cleanser. Based on this, the method of culturing the microbial suspension with a cleanser together becomes a tool for observing the inhibitory effect on microorganism growth. In this study, *C. albicans*, most commonly found on the denture surface, and *S. mutans*, found on the surface of the orthodontic appliance, were selected as representative microorganisms for evaluation of antimicrobial efficacy[21,24,28,30,36-40]. Both strains showed CFU reduction rates of close to 100% after at least 10 min incubation with cleansers.

In addition, when the removable appliance is immersed in a cleanser, microorganisms are killed or removed from the appliance surface. The method of quantifying the microorganisms removed from the specimen surface is a tool to observe the antimicrobial effect of the cleanser. In this study, microorganisms were attached to the plastic (thermoplastic polymer sheet) surface in consideration of international standards and clinical conditions. At this time, a rough surface was formed on the surface of thermoplastic sheets and used as a specimen for the efficacy evaluation, referring to prior studies that showed that microorganisms attached better to rough surfaces than to smooth[41,42]. Compared to the smooth surface, the adhesion of a large number of microorganisms to the rough surface was also verified by our SEM results (Fig. 1).

The antimicrobial effect of the cleansers was greater on the specimen surface with *S. mutans* compared to *C. albicans*. As the application time of the cleansers increased, the rate of microbial removal also increased. In addition, distilled water and sodium hypochlorite solution were set as negative and positive controls, respectively, and the validation of the antimicrobial efficacy test was verified by comparative evaluation with commercially available cleanser products. The method implemented in this study was also able to confirm repeatability and reproducibility through repeated experiments. Through the evaluation of antimicrobial efficacy established in this study, it was determined that the seven commercially available cleansers used are effective in removing and inhibiting the growth of microorganisms attached to the denture or orthodontic appliance.

Evaluation of the cleansing effect of dentures or orthodontic appliance cleansers should be performed by a method capable of objectively and quantitatively evaluating the effect of removing stains remaining on the surface of the denture or orthodontic appliance. According to previous studies observing stain removal properties, researchers have designed and considered types of specimens (microplate, clear poly(methylmethacrylate) sheet, heat-cured acrylic resin), pre-staining of specimens, and assessment of the results[26,31,32,43,44]. Thus, in this study, a method for evaluating the stain removal efficacy of cleansers was established by applying methods from previous studies. As a result, every cleanser showed a stain removal rate of 70% or more at all application times, which was significantly higher than the negative control, with a cleaning rate of 50%. In addition, as the application time increased, the stain removal rate was not significantly different from that of the positive control. The plate reader used in evaluating the stain removal rate is a device that is easy to use and can quantify stains remaining after the cleansing procedure[32]. Therefore, it could be determined that commercially available cleansers have the effect of removing stains deposited on the surface of the denture or orthodontic appliance.

As soon as the surface is in contact with the protein solution, it will be covered almost immediately by an initial protein layer[45]. In the intraoral environment, this layer is called the salivary pellicle[46]. Pellicle formation is a very selective process in which only a fraction of the salivary protein adsorbs onto the tooth surface. The pellicle also has a pathological relevance and serves as a mediator between the solid surface in the oral cavity, saliva, and microorganisms. Therefore, the pellicle contributes to microbial adhesion and thus the formation of pathogenic biofilms, called dental plaque[47,48].

Some denture or orthodontic appliance cleansers include enzymes in their constituents, so the proteolytic or protein removal effect of the product has been described by the manufacturer[49-51]. The measurement of protein removal and the amount of protein remaining on the surface of the denture or orthodontic appliance after application can be a tool for evaluating the protein removal efficacy of cleansers[27,52]. Protein assays should be sufficiently sensitive to quantify proteins in highly dilute solutions and free from interference by cleanser components[27,53]. In a previous study, dye-binding techniques were used to determine the area of plaque extension on dentures and had been used as the basis for quantitative assays. However, the staining procedure may not allow full penetration of the dye into the plaque under the mild conditions necessary to maintain plaque

integrity[54,55]. Thus, dye-binding does not provide a truly quantitative evaluation of denture plaque before or after cleanser application. In another approach by Connor *et al*., to evaluate plaque removal by a cleanser, SEM was selected to visualize plaque accumulation[56]. Through this approach, it was not possible to observe a specimen both before and after cleanser application because the SEM preparation procedure is irreversible. This technique also requires questionable quantification of the efficacy of denture cleansers. Based on these previous studies, although there are many different experimental settings to determine the protein amount, it is difficult to quantify, reproduce, and easily evaluate a small amount of protein. For the quantification of protein remaining on the surface of medical devices, international standards ISO/TS 15883-5:2005 and literature describe the preparation of proteins, inoculation of test pieces, detection, and analysis of residual protein[17,34,35,52,57,58]. In these studies, it was shown that certain impurities in industrial-grade chemicals were used to evaluate the cleansers using a sensitive spectrophotometric assay. In our study, BSA was chosen because of its structural similarity to human serum albumin[59]. Furthermore, it is an important blood plasma protein that is also present in the salivary pellicle[45,60-62]. To simulate the adsorption of proteins on the surface of the denture or orthodontic appliance, the BSA solution was applied to a thermoplastic polymer sheet and then dried to deposit BSA on the sheet surface. The BCA assay was then applied to quantify the BSA remaining on the surface after applying the cleansers. For the measurements in this study, the Pierce™ BCA protein assay kit (Thermo Scientific) was used[63]. This method combines the well-known reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium (the biuret reaction) with highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+1}) using a unique reagent containing BCA. The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbance at 562 nm, and the color intensity correlates directly with the number of protein molecules in the sample. This method does not require special properties of the sample, such as high reflectivity or conductivity, and turns the BCA assay into a valuable tool to evaluate a wide spectrum of different substrate materials[64-66].

For the validity of the protein removal efficacy test, negative and positive controls were evaluated together and compared with the denture or orthodontic appliance cleansers. We observed that as the application time of the cleanser increased, the protein removal rate also increased for all cleansers. The negative control also showed an increase in the protein removal rate over time, but it was significantly lower than that of denture or orthodontic appliance cleansers. This is presumably a phenomenon caused by the dissolution of the remaining BSA on the surface in distilled water over time. In contrast, the positive control showed a protein removal rate of 100% at all application times. Therefore, it can be determined that commercially available denture or orthodontic appliance cleansers have the effect of removing proteins deposited on the surface of the denture or orthodontic appliance. The quantification test of the protein remaining on the specimen surface also confirmed that the concentration of attached protein was close to the application concentration (2,000 μg/mL) of the BSA standard solution before the cleansers were applied. The amount of protein remaining on the sheet surface decreased significantly as the application time of the cleansers increased, which can be attributed to proteolysis or protein removal from the specimen surface by the cleansers. The BCA assay for the protein removal efficacy test described here overcomes the limitations of previous studies. This method provides a quantitative measurement of the remaining protein on removable appliances both before and after application with commercially available cleansers. Quantification of protein content is correlated with residual protein on the surface of the specimen and can be used to estimate the cleanser's efficiency for protein removal.

Considering the continuously increasing demand for removable dentures or orthodontic appliances and their cleanser market trends, studies on the effectiveness of cleanser products through objective and standardized methods are essential. In addition, effective products should be distributed to the market through a study on the efficacy evaluation of cleanser products by simulating the clinical situation. In this study, the experiment was conducted only with some commercially available cleanser products, and the evaluation was performed only *in vitro*. Some *in vitro* studies used saliva pre-treated specimens before incubation with microbial suspension, these pre-treatment is known to be an important factor to simulate clinical situations[67]. However, we did not pre-treat the salivary protein adsorption in order to prevent the influences of any other factors. Nevertheless, for antimicrobial and stain removal efficacy test, it is necessary to simulate the clinically relevant conditions, such as an acquired salivary pellicle formation derived from healthy volunteers. In addition, further research is needed to complement these limitations by analyzing the correlation between the *in vivo* and *in situ* results and the current *in vitro* results. Nevertheless, the efficacy evaluation methods developed in this study were meaningful research methods for the assessment of cleaner effectiveness. These methods may be useful in evaluating cleansers by addressing the limitations of previous studies.

CONCLUSION

This study developed reproducible *in vitro* methods to evaluate the antibacterial and antifungal efficacy, stain removal efficacy, and protein removal efficacy of commercially available denture or orthodontic appliance cleansers according to their application time.

All seven of the commercially available denture or orthodontic appliance cleansers used in this study showed antimicrobial efficacy against *C. albicans* and *S. mutans*, as well as stain and protein removal efficacy; while these

efficacies improved with increasing application time, parallel *in vivo* or *in situ* studies are needed to determine the extent to which extrapolation of the *in vitro* data is possible. Based on this evaluation, a predictive model of clinical outcomes can be developed by researchers and manufacturers and applied to the hygiene management of removable dentures or orthodontic appliances.

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