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ORIGINAL RESEARCH

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Antimicrobial effect of two fluoride-releasing adhesive tapes on *Streptococcus mutans* biofilm

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Abstract

Fluoride-releasing adhesive tapes have been developed as a new fluoride delivery agent. However, application as caries prevention agents remains underexplored. This study aimed at evaluating the antimicrobial activity of two fluoride-releasing adhesive tapes against *S. mutans* biofilm. Two polyvinyl alcohol (PVA) tapes were investigated: (i) a fluoride-PVA (F-PVA) tape, (ii) a pullulan incorporated F-PVA (PF-PVA) tape. *S. mutan* strains were cultured and treated with the tapes. Antimicrobial effects were evaluated using the agar diffusion test, field-emission scanning electron microscopy (FE-SEM), and confocal laser scanning microscopy (CLSM). F-PVA tapes showed higher inhibition-zone diameters than PF-PVA at 48 h and 72 h. However, there were no significant differences ($p > 0.05$) between the effects of F-PVA and PF-PVA. The bio-volume of *S. mutans* and extracellular polymeric substances significantly decreased in the F-PVA tapes than in the PF-PVA tapes ($p < 0.05$). FE-SEM micrographs revealed less *S. mutans* colonization in F-PVA. F-PVA exhibited better antimicrobial activity against *S. mutans* than PF-PVA.

Keywords

Cariogenic biofilm; Dental caries; Fluoride; Polyvinyl alcohol tape; Pullulan

1. Introduction

Dental caries constitute an infectious disease with a high prevalence in the primary dentition [1]. The progression of dental caries depends on the balance between demineralization and remineralization [2]. According to the "Caries Balance" concept, the fate of carious lesions is determined by the net effect of protective factors and pathological factors [3]. Fluoride, one of the protective elements, promotes enamel remineralization and reduces enamel demineralization [4]. The increased fluoride content facilitates remineralization within the surface region of enamel caries lesions [5]. Furthermore, fluoride has been reported to influence the virulence of bacterial biofilms and their acid production [6], effectively inhibiting the development, adhesion and acid synthesis of *S. mutans* biofilms [7]. In particular, fluoridation remains considered as a preventive treatment following the molar incisor hypo-mineralization (MIH) diagnosis [8].

Fluoride can be delivered from various sources. Today, fluoride varnishes are widely used as preferred sources. Fluoride varnishes were approved by the US Food and Drug Administration (FDA) in 1994 for commercial use and constitute the most widely used and effective fluoride treatment method available to dental professionals [9, 10]. However, fluoride varnishes have some drawbacks. The natural resin used within fluoride varnishes to facilitate adhesion to enamel may cause temporary tooth discoloration. Moreover, the unpleasant taste and surface texture of fluoride varnish may be unpopular with children. In addition, varnishes cannot maintain fluoride concentrations in the oral cavity due to the continuous flow of saliva [11, 12].

An ideal vehicle for fluoride should be easily applicable, efficient and biologically stable. It should provide strong adhesion to enamel and allow for a continued release of fluoride. To meet these criteria, fluoride-polyvinyl alcohol (F-PVA) tapes were developed by supplementing a polymer-based substrate with nano-sized fluoride particles [13]. F-PVA tape covers all teeth in both arches with approximately 33% of the amounts of fluoride contained in fluoride varnish [14]. The application of F-PVA exhibits less toxic potential [13]. However, previous literature demonstrated that F-PVA did not showed more ability to promote remineralization and inhibit demineralization than fluoride varnish [14–16], probably due to limited enamel retention potential [14, 17]. Therefore, F-PVA may exhibit low potential to sustain fluoride concentrations in the oral environment for a long time by gradual fluoride release.

A novel variant of F-PVA known as pullulan to F-PVA (PF-PVA) was developed to enhance the adhesion of fluoride, incorporating an additional layer of pullulan onto the F-PVA adhesive film. PF-PVA represents a dual-layer adhesive film that exhibits enhanced properties compared to the conventional F-PVA [18]. Pullulan is a biocompatible material often used as a food additive to increase the stickiness and viscosity and enhance the properties and texture [19]. Pullulan has proven biocompatibility as a drug vehicle with adhesive abilities [20].

Previous studies [13–16] on fluoride adhesive films have predominantly concentrated on their remineralization and dental caries prevention effects. Nonetheless, less attention was given to the antibacterial impact of fluoride adhesive films against caries-causing bacteria [13]. One of the fundamental mechanisms through which fluoride prevents dental caries is by inhibiting bacterial colonization and metabolism. *S. mutans* particularly contribute to the initiation and progression of dental caries and is mainly target by the fluoride adhesive films. Therefore, the purpose of the present study was to evaluate the antibacterial effects of F-PVA and PF-PVA tapes against *S. mutans* biofilm. The null hypothesis of this study was that there is no difference in antimicrobial effect on *S. mutans* between the investigated tape groups.

2. Materials and methods

The agar diffusion test was conducted to measure the inhibitory zones [21]. Confocal laser scanning microscopy (CLSM) (Molecular Probes, Eugene, OR, USA) and scanning electron microscopy (SEM) were utilized to observe and identify the formation and surface morphology of the biofilm [22, 23].

2.1 Preparation of F-PVA tape

F-PVA tape was prepared according to a method previously described in [14]. After dissolving 10 g of PVA and 5 g of polyacrylic acid in 85 g of distilled water, the contents were stirred for approximately 2 h at 85 °C. Subsequently, 3 mL of polyethylene glycol (Sigma-Aldrich Inc., St. Louis, MO, USA) was used as a plasticizer, and 0.95 g of sodium fluoride (NaF; 5%) was added, and the stirred for 2 h. After pouring the cross-linked solution evenly on the surface of the glass plate, an applicator was used to create a uniform thickness of 80 μm. F-PVA tape development was completed through a drying process (Fig. 1).

2.2 Preparation of PF-PVA tape

Two wt% of polyethylene glycol (Sigma-Aldrich Inc., St. Louis, MO, USA) were added to serve as a plasticizer to 10 wt% of pullulan powder (Sigma-Aldrich Inc., St. Louis, MO, USA). The mixture was subsequently stirred for 2 h. Forty μm of Pullulan adhesive film solution was applied to dried F-PVA tape. Subsequently an applicator was used to create another 40 μm layer for a total thickness of 80 μm. The PF-PVA tape was dried for 24 h at 40 °C to achieve a complete removal of all moisture from the tape.

2.3 Experimental groups

The experimental samples were divided as follows:

(i) Control group: PVA tape with no fluoride supplementation.

(ii) F-PVA group: PVA tape with 5% NaF.

(iii) PF-PVA group: F-PVA tape with an addition of a pullulan layer.

2.4 Agar diffusion test

An agar diffusion test was performed using a *Mitis salivarius* agar plate (MS agar: MB cell, Seoul, Korea). The tapes were placed on top of the plate and gently pressed after inoculating the MS agar plate with *S. mutans* (1×10^8 CFU/mL), the plate was cultured at 37 °C and ImageJ program (https://imagej.nih.gov/ij/download.html) was used to identify the zone of inhibition (ZOI) after 24, 48 and 72 h. Six independent experiments were performed for each group.

2.5 Bacterial culture and biofilm formation

S. mutans UA159 was incubated at 37 °C in brain heart infusion (BHI; Difco Laboratories, Detroit, MI) medium. The sample was placed in a 24-well plate containing 1 mL of artificial saliva 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, 30 mM potassium chloride (KCl), 4.0 mM potassium dihydrogen phosphate (KH_2PO_4), 0.2 mM magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$), 0.7 mM calcium chloride ($CaCl_2$), 0.3 mM sodium azide (NaN_3, pH 7.4 [24]) for 1 h for biofilm development and the film was subsequently placed on top of the sample. Afterward, *S. mutans* (1×10^6 CFU/mL) strains were transferred to the 24-well plates containing the samples covered with the tapes in 1 mL of BHI medium containing 1% sucrose (BHIS). The bacteria were incubated for 48 h at 37 °C and the medium was replaced daily.

2.6 Confocal laser scanning microscopy (CLSM)

The assessment of the viability of *S. mutans* in biofilms was conducted using CLSM analysis under LIVE/DEAD BacLight stain (Molecular Probes, Eugene, OR, USA), as previously described with some modifications [25]. *S. mutans* (1×10^6 CFU/mL) suspension was incubated for 48 h at 37 °C in a 24-well plate containing a BHIS medium with 1 μm of Alexa Fluor 647-labeled dextran adhesive (Molecular Probes, Eugene, OR). *S. mutans* in the samples were treated with 2.5 μm SYTO 9 green-fluorescent nucleic acid stain (480/500 nm; Molecular Probes) for 30 min at room temperature. An LSM 510 META microscope (Carl Zeiss, Jena, Germany) was used to acquire six image stacks (512 × 512-pixel tagged image file format) per biofilm experiment. The quantification of the bio-volumes of bacteria and extracellular polymeric substances (EPS) was performed using COMSTAT (https://www.comstat.dk; Technical University of Denmark, Kongens Lyngby, Denmark). Three independent experiments were performed on each group.

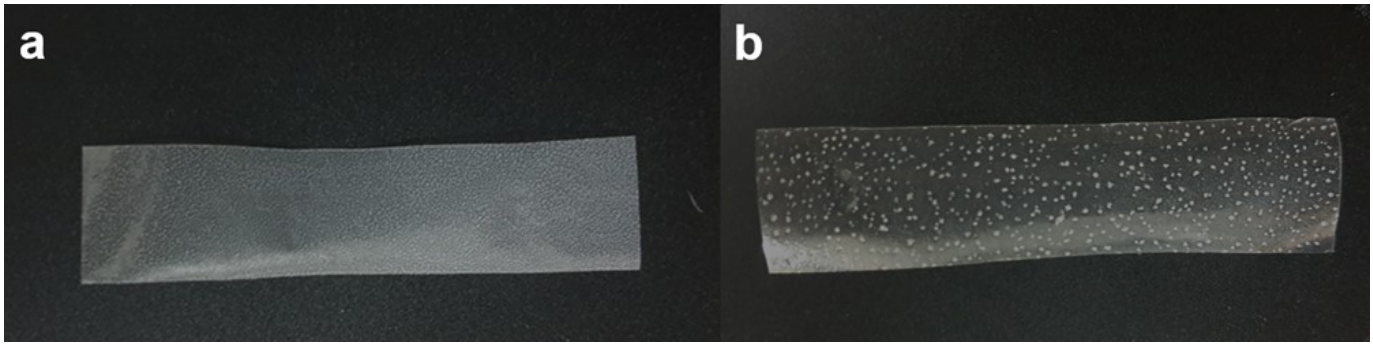

FIGURE 1. PVA tapes used in this study. (a) F-PVA, (b) PF-PVA.

2.7 Field-emission scanning electron microscope (FE-SEM)

FE-SEM analysis was performed to investigate the effects of tapes on the biofilm formation with some modifications, as previously described [26]. First, the samples were placed in a 24-well plate containing phosphate buffer saline (PBS) with 2.5% glutaraldehyde and 4% paraformaldehyde (Sigma-Aldrich, Saint Louis, MO) and left overnight at 4 °C. The samples were washed twice with PBS and dehydrated using ethanol (25~100%). The samples were dried and gold-coated, followed by observation using a field-emission scanning electron microscope (FE-SEM, Hitachi, Tokyo, Japan).

2.8 Statistical analysis

The experimental data used in this paper was analyzed using the SPSS version 20.0 (SPSS Inc., Chicago, IL, USA). The non-parametric statistical analysis was used in this study due to the sample size that did not satisfy the normality requirements. The Kruskal-Wallis test was used to analyze the original data, and the Mann-Whitney test was used for *post-hoc* analysis. Statistically significant differences were defined at $p < 0.05$.

3. Results

3.1 Zone of inhibition (ZOI)

There was no significant difference in ZOI diameters between F-PVA and PF-PVA groups after 24 h. After 48 h and 72 h, the growth of *S. mutans* was significantly inhibited in the F-PVA group compared to the PF-PVA group (Fig. 2, Table 1). However, there were no significant differences in ZOI diameters within both F-PVA and PF-PVA groups.

3.2 CLSM image analysis

The viability of *S. mutans* in biofilms after treatment with tapes was analyzed using CLSM and the Live/Dead assay kit (Fig. 3). Bio-volumes of *S. mutans* and EPS showed a significant decrease in F-PVA and PF-PVA groups compared to the control group ($p < 0.05$). Moreover, F-PVA was highly effective in reducing bio-volumes of *S. mutans* and EPS than PF-PVA ($p < 0.05$).

3.3 FE-SEM analysis

Fig. 4 shows the FE-SEM micrographs of *S. mutans* biofilm. Compared to the control, the biofilm showed lower density and loosely arranged structure in F-PVA and PF-PVA groups. The biofilm in the F-PVA group showed a more porous and loose arrangement than that in the PF-PVA group. The bacteria developed in the F-PVA group showed relatively low colonization.

4. Discussion

Recent research has attempted to address the control of mucosal drug release *via* biocompatible polymer vehicles [20, 27, 28]. A previous study demonstrated that nano-sized calcium fluoride exhibited a better response than macro-size fluoride due to differences in solubility. Thus, the tapes used in this study were developed with the concept of mucoadhesion and nano-sized fluoride incorporation [13].

Numerous ongoing studies aim at optimizing the physical attributes, dissolution rate and adhesion properties of polymeric films used for drug delivery in the oral cavity, including fluoride and fenestration agents [29–33]. However, the majority of these investigations have been centered around single-layer adhesive films, with limited attention to double-layer adhesive films.

Primary mechanisms of fluoride against dental caries involve effects on oral cariogenic bacterial colonization and enamel remineralization/demineralization [4, 5]. An *in-vivo* study showed that the metabolism of *S. mutans* and *Lactobacillus* was inhibited by fluoride [34]. In an acidic environment, fluoride invades bacterial cells and promotes cytoplasmic acidification, thus inhibiting bacterial metabolism [35]. Moreover, fluoride inhibits *S. mutans* adhesion [36, 37]. Therefore, we evaluated the antimicrobial activity of two types of films on *S. mutans* biofilms [18].

Agar diffusion test revealed that both tapes showed inhibitory activity on *S. mutans* biofilm during the experimental period. This finding is corroborated by the results of a previous study in which various fluoride varnishes resulted in increased ZOI diameters against *S. mutans* biofilms after 24 h of exposure [38]. This phenomenon can be attributed to higher fluoride concentrations (5%). A previous study [39] showed that fluoride concentrations >225 ppm potentially inhibited the-colonization of *S. mutans* species. Moreover, NaF exhibits bactericidal activity against oral bacteria at 12,500 ppm [40].

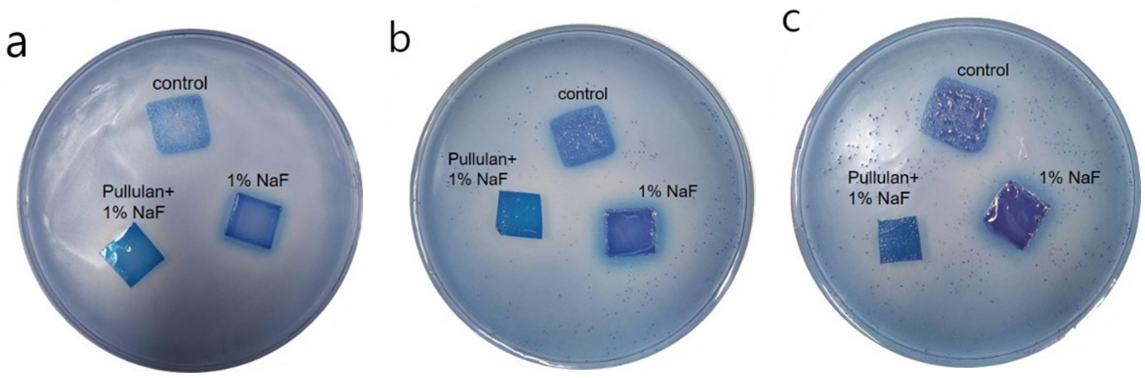

FIGURE 2. Sample plate of agar diffusion tests. (a) 24 h, (b) 48 h, and (c) 72 h of an agar plate showing zone of inhibition. F-PVA showed the highest growth inhibitory zone after 48 hours. NaF: sodium fluoride.

TABLE 1. The zone of inhibition (ZOI) diameters of the tapes against *S. mutans*.

	Control	F-PVA	PF-PVA
After 24 h	ND	$24.04 \pm 3.07^{a,A}$	$21.39 \pm 1.05^{b,A}$
After 48 h	ND	$26.25 \pm 2.72^{a,A}$	$21.39 \pm 1.07^{b,B}$
After 72 h	ND	$25.32 \pm 3.19^{a,A}$	$21.37 \pm 1.07^{b,B}$

ZOI diameters were presented as mean ± SD (mm). Different small case letters indicate statistically significant difference within the same column ($p < 0.05$) and different capital case letters indicate statistically significant difference within the same row ($p < 0.05$). ND: Not detectable; F-PVA: fluoride-polyvinyl alcohol; PF-PVA: pullulan incorporated fluoride-polyvinyl alcohol.

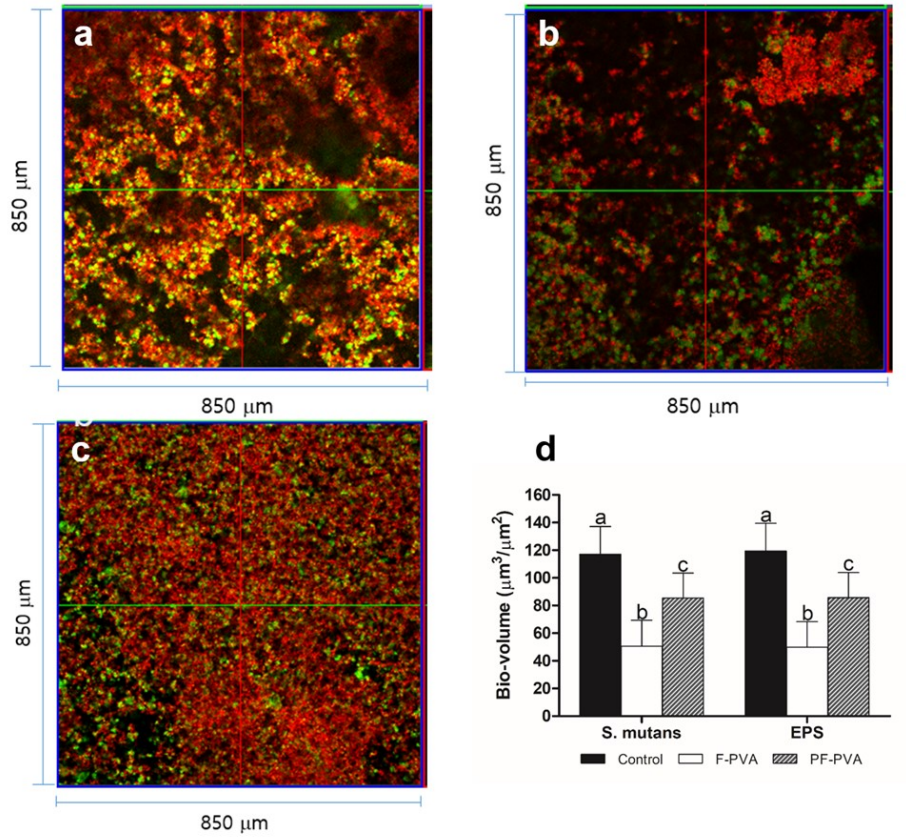

FIGURE 3. CLSM analysis. (a) A representative CLSM image of *S. mutans* of control, (b) A representative CLSM image of *S. mutans* after treatment with F-PVA tape, (c) A representative CLSM image of *S. mutans* after treatment with PF-PVA tape, (d) Bio-volume of *S. mutans* biofilms. The different case letters indicate statistical differences between groups ($p < 0.05$). EPS: extracellular polymeric substance. F-PVA: fluoride-polyvinyl alcohol; PF-PVA: pullulan incorporated fluoride-polyvinyl alcohol.

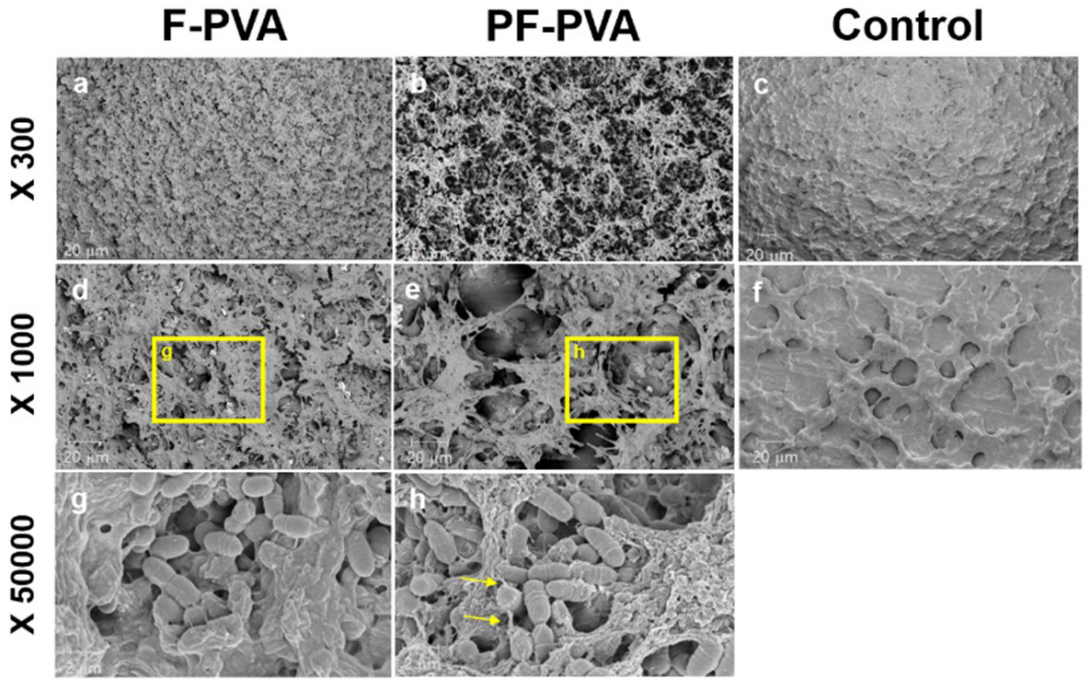

FIGURE 4. FE-SEM images of *S. mutans* biofilm. (a, d and g) F-PVA; (b, e and h) PF-PVA; (c and f) Control group. Note that extracellular polymeric substances (EPS) are visible in PF-PVA (yellow arrows). (g) and (h) are high magnification views of the square regions in (d) and (e). The magnification of the images was 300× (a–c), 1000× (d–f), and 50000× (g and h). F-PVA: fluoride-polyvinyl alcohol; PF-PVA: pullulan incorporated fluoride-polyvinyl alcohol.

Both CLSM analysis and FE-SEM analysis provided similar findings. Both tapes significantly decreased the bio-volume of *S. mutans* in biofilms and EPS compared to the control. EPS is the basic component of biofilms, consisting of polysaccharides, proteins and extracellular DNA [41]. Cells within EPS have a higher resistance to antibacterial effect than other cells [42]. FE-SEM images showed that both tapes altered surfaces of the biofilms compared to the control in lower magnification images. Moreover, different cluster arrangements were observed in higher magnification images of both tapes compared to the control group. These findings support that both tapes exhibit antimicrobial activity against *S. mutans* biofilms.

Pullulan has structural flexibility and a uniform and unique linkage pattern of 9 hydroxyl groups on glucopyranose rings, exhibiting distinct film and fiber-forming characteristics absent in other polysaccharides [43]. It is widely used in drug delivery systems, tissue engineering, wound healing and oral care products due to these characteristics [43, 44]. Studies using cross-linked pullulan nanoparticles for drug delivery systems have reported that pullulan nanoparticles are non-toxic to cells [45, 46].

The findings in this paper confirmed that PF-PVA had lower antimicrobial activity against *S. mutans* than F-PVA, thus the null hypothesis was rejected. In a previous study, comparing the elution rates of the two polymeric adhesive films and found that more elution occurred from F-PVA after 1 h of tape application [18]. In this experiment, it was observed up to 72 h, which suggests that the higher fluoride elution from F-PVA may have contributed to the antibacterial effect. Furthermore, the difference in antimicrobial activity may be due to differences in relative reactivity to fluoride ions between two tapes. Pullulan might potentially absorb fluoride ions and therefore interfering with fluoride binding mechanism to biofilms. It is worth noting that pullulan is recognized for its remarkable biocompatibility and potential use as an adsorbent [47]. It has been successfully employed in the removal of fluoride from drinking water [48]. In this study, its fluoride adsorption capacity is believed to have led to a reduction of the amount of fluoride eluted into the external environment.

As pullulan has several hydroxyl groups, there may be an ion exchange reaction between fluoride and hydroxyl groups, resulting in large amounts of fluoride adsorption [49, 50]. A previous study evaluating the defluoridation property of calcined magnesia/pullulan composite demonstrated that the absorption ability of fluoride ions synergized with addition to pullulan [47].

This study has some limitations. First, the investigation using large size of samples could have improved the statistical accuracy of the findings. Secondly, the evaluation of the antibacterial effect was limited to *S. mutans*, thus further investigations with a broader spectrum of bacteria is suggested. Despite these limitations, our study successfully demonstrated the antimicrobial activity of a novel fluoride delivery agent. The consistency of results across three different experiments reaffirms the antibacterial efficacy of both fluoride tapes.

Future experiments should focus on assessing the biostability of F-PVA and FP-PVA, as this is pivotal for the commercial viability of such tapes. Additionally, conducting *in vivo* studies would be valuable to gauge the practical implications and potential of these tapes in real oral conditions.

5. Conclusions

In conclusion, this study confirmed the superior antibacterial efficacy of F-PVA against *S. mutans* compared to PF-PVA augmented with pullulan. These results suggest that, in exchange for increased adhesive capability and user convenience, PF-PVA suffered some loss of anticarious efficacy when using fluoride agents in conjunction for preventing dental caries.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

AUTHOR CONTRIBUTIONS

OHN and MKJ—designed the research study. OHN and MKJ—performed the research. JHS and SRJ—provided help and advice on the research. OHN, TYP and MKJ—wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was conducted following the Declaration of Helsinki. The study protocol was reviewed and approved by the Institutional Review Board of the Chosun University Dental Hospital, Seoul, Korea (CUDHIRB 2102-001).

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Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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