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## Effect of *S. Mutans* and *S. Sanguinis* on Growth and Adhesion of *P. Gingivalis* and Their Ability to Adhere to Different Dental Materials

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Data Collection B  
Statistical Analysis C  
Data Interpretation D  
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**Background:** Caries and periodontal diseases are caused by the biofilm formed by caries- and periodontal disease-related bacteria. Specific biofilms could be formed on different filling materials in oral cavity. Thus, to explore the inhibition effect of restorative filling materials on biofilm formation is of great significance in the treatment of caries and periodontal disease.

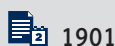
**Material/Methods:** The supernatants of *S. mutans*, *S. sanguinis*, and *P. gingivalis* suspension were combined with BHI broth. After 24 h, the live *P. gingivalis* number was calculated by colony counting and the biofilm was monitored by fluorescence microscopy. To test the adhesive ability of *S. mutans* and *S. sanguinis* on different dental materials, the biofilm was formed on different dental materials and then the bacterial number was calculated by using a Spectramax 250 microplate reader at OD 550, and the adhesive ability of *S. mutans* and *S. sanguinis* on different dental materials was analyzed by scanning electron microscopy.

**Results:** The growth and biofilm formation of *P. gingivalis* was significantly inhibited by *S. mutans* and *S. sanguinis* supernatants ( $P < 0.05$ ). All groups except the zinc phosphate cement group (B) exerted a strong inhibitory effect on the biofilm formation of *S. mutans* and *S. sanguinis* ( $P < 0.05$ ).

**Conclusions:** The supernatants of *S. mutans* and *S. sanguinis* significantly inhibited the growth and biofilm formation of *P. gingivalis*, and the adhesive ability of *S. mutans* and *S. sanguinis* are different on different dental materials. These results provide useful information on dental caries, periodontal disease, and dental materials.

**MeSH Keywords:** **Biofilms • Biomedical and Dental Materials • Dental Caries • Periodontal Diseases**

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## Background

Caries and periodontal diseases are the most common bacterial diseases of humans and are caused by changes in the oral microflora. Dental plaque biofilms, which can be formed on enamel, filling material surfaces, and implants, are one of the key etiological factors of dental caries and periodontal diseases, and is also a popular research topic in oral microbiology and ecology [1].

With increasing aesthetic requirements, restorative filling materials that match with the color of natural teeth have been developed. The filling materials include nanoparticle resin composites, zinc phosphate cement, and glass ionomer cement [2]. However, because of their improved mechanical properties, there are at present certain disparities regarding the ideal material for tooth repair [3]. Secondary caries occur on the edge of the restoration when dental plaque biofilms are formed [4]. Different filling materials in the mouth may form specific biofilms [5]. Thus, the inhibitory efficiency of restorative filling materials has attracted more and more research attention.

*S. mutans* and *S. sanguinis* are the main bacteria that form biofilms and cause caries [6], and *P. gingivalis* is one of the main bacteria that forms biofilms and causes periodontal disease [7]. Therefore, control of biofilm formation is the key to preventing caries and periodontal disease. The relationship between periodontal disease and caries-causing bacteria has been an important topic in the field of oral microbiological research [8]. In the present study, in order to explore the interaction between *S. mutans*, *S. sanguinis*, and *P. gingivalis* and evaluate the adhesive ability of *S. mutans* and *S. sanguinis* on different dental filling materials, we compared the inhibitory effects of supernatants of *S. mutans* and *S. sanguinis* on *P. gingivalis* growth and biofilm formation and tested the adhesive ability of *S. mutans* and *S. sanguinis* on different materials.

## Material and Methods

### Strains and conditions

The strains used in this study were *S. mutans* ATCC 25175, *S. sanguinis* ATCC 10556, and *P. gingivalis* ATCC 53977. These 3 strains were incubated on Brian Heart Infusion agar at 37°C under microaerophilic conditions. The bacteriostatic and biofilm experiments were conducted in Brian Heart Infusion broth and Brian Heart Infusion agar (Oxoid, UK).

### Preparation of sterile supernatants

The mixtures of 1 ml of *S. mutans* (Sm) and *S. sanguinis* (Ss) ( $3 \times 10^8$  CFU/ml) and 4.0 ml of BHI broth were incubated under

microaerophilic conditions for 48 h at 37°C. Then, the culture was centrifuged for 15 min at 2100 rpm to remove all cells. After centrifugation, the supernatant was filtered through 0.22- $\mu$ m cellulose nitrate filters (Millipore, USA) and stored at 4°C [9].

### Inhibitory effect of sterile supernatants on *P. gingivalis* growth

*P. gingivalis* was first grown in BHI broth at 37°C for 24 h and then was suspended in saline to form a concentration of 0.5 MCF ( $1.5 \times 10^8$  CFU/ml) suspension. Then, 250  $\mu$ l of the Sm and Ss supernatants and 250  $\mu$ l of the *P. gingivalis* suspension were combined with 0.35 ml BHI broth. The cultures were incubated under microaerophilic conditions at 37°C for 24 h. The BHI broth was used in the control instead of Sm and Ss supernatants. After 24 h, the cultures were vortexed for 1 min, diluted to  $10^{-4}$ , and the colony number was determined.

### Biofilm tests

#### Inhibitory effects of sterile supernatants of *S. mutans* and *S. sanguinis* on *P. gingivalis*

For the biofilm formation of *P. gingivalis*, the Pg culture was incubated in BHI broth under aerophilic conditions [10] (10% CO<sub>2</sub>, 5% H<sub>2</sub>, 85% N<sub>2</sub>), for 24 h at 37°C. Then, the concentration of *P. gingivalis* suspension was adjusted with saline to 0.5 MCF ( $1.5 \times 10^8$  CFU/ml). Individually, 10 ml of BHI broth and a sterile coverglass (18 mm diameter) were added in the sterile Petri dishes. Then, 100 ml of the Pg suspension and 500  $\mu$ l of the Sm and Ss supernatants were mixed in the dishes. The BHI broth was used as the control groups. All dishes were incubated under aerophilic conditions at 37°C for 24 h.

After 24 h, the coverglasses in dishes were washed with phosphate-buffered saline (PBS) 3 times and fixed with 2.5% glutaraldehyde for 1 h. After fixation, the coverglasses were stained using the BacLight Live/Dead staining kit (Invitrogen). The images of the biofilms were acquired using a Nikon 80i fluorescence microscope at an excitation wavelength of 488 nm (green fluorescence). Eight images per field per sample of the biofilm were acquired randomly and the experiment was independently repeated 3 times. The biofilm images were analyzed by using Image-Pro Plus 6.0 software and expressed as the integrated optical density (IOD) [11].

#### Adhesive ability of *S. mutans* and *S. sanguinis* on different dental materials

Sixty specimens containing 4 repair materials and three 3-root canal sealers were divided into 7 groups according to the type of composite used, as follows:

**Table 1.** Viable Pg cells in mixed culture with the supernatants of Sm, Ss. Viable Pg, 10<sup>8</sup> CFU/ml (means ±SD).

Pg concentrations	Sm supernatants	Ss supernatants	Control
1.5	3.33±1.32*	3.65±0.74*	7.01±2.45
3	2.55±0.65*	1.84±0.64**	5.20±1.44

\* P<0.05; \*\* P<0.01 compared with controls (Dunnett's two-sided *t*-test).

- Group A: Nanoparticle resin composite Filtek Z350 (3M ESPE, USA), shade A3;
- Group B: Zinc phosphate cement (Ultradent, South Jordan, UT, USA), shade A3;
- Group C: Glass ionomer cement (3M ESPE, St. Paul, MN, USA), shade A3.
- Group D: Infiltrant resin (ICON®-Infiltrant; DMG shade A3.
- Group E: Mineral Trioxide Aggregate (MTA) (ProRootMTA; Dentsply).
- Group F: iRoot SP root canal sealer (Innovative BioCreamixInc, Canada).
- Group G: iRoot BP root canal sealer (Innovative BioCreamixInc, Canada).
- Group H: iRoot FS root canal sealer (Innovative BioCreamixInc, Canada).
- Group I: AH Plus sealer (Dentsply).

The composites were inserted at a single increment into a cylindrical metal matrix of 1-mm height with 10-mm diameter, and placed on a glass plate. After curing, the material was removed with the aid of a spatula. The composite surfaces were ground with 1200-grit silicon carbide abrasive paper (3M ESPE, St. Paul, MN, USA) with water-cooling for surface standardization and then disinfected using ultraviolet illumination for 2 h [12].

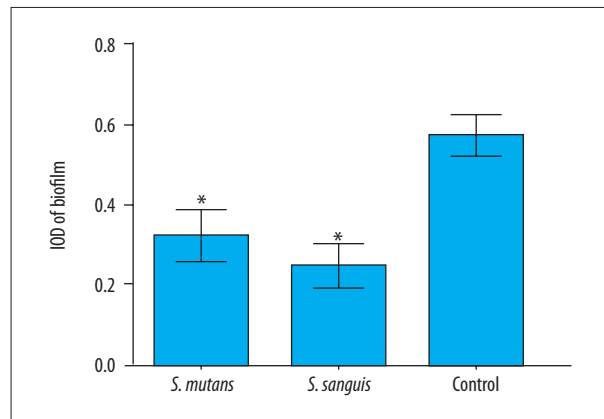
Biofilm formation was conducted using the methods of Stoodle et al. [13]. In brief, the material from the 3 groups was placed in 16-well plates, and 2-ml BHI and 100-µl MS suspension (1.5×10<sup>8</sup> cfu/ml) was added to each hole. The holes were incubated under anaerobic conditions for 24 h.

#### Assessment of bacterial adhesion

After 24 h, the coverglass was washed with PBS 3 times to remove unattached cells and were then sonicated in 10 ml PBS for 6 min to detach adherent bacteria on the biomaterial surfaces. After sonication, the suspensions were added in the tube and the OD at 550 nm (OD 550) was measured using a Spectramax 250 microplate reader [14].

#### Scanning electron microscopy (SEM) of biofilms

The biofilms were fixed with 2.5% glutaraldehyde, dried at 37°C for 24h, and then the biofilms were coated with gold



**Figure 1.** The adhesion/biofilm formation (IOD) of Pg grown in the presence of Sm and Ss supernatants. Data are expressed as the mean ±SD, \* P<0.05.

and examined under a scanning electron microscope (Hitachi SU-70) in high-vacuum mode at 3 kV. The SEM images were analyzed using Image J software to measure the biofilm area (in pixels) [15,16].

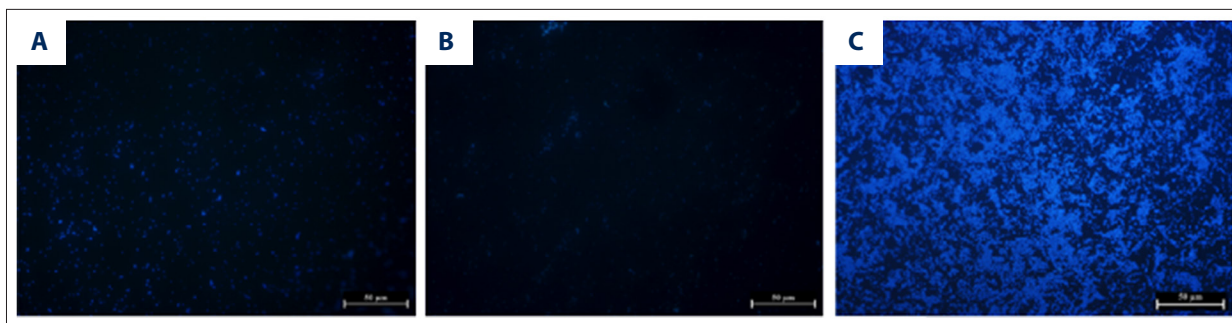
#### Statistical analysis

SPSS 14.0 software was used for statistical analysis by a one-way analysis of variance (ANOVA) and Dunnett's two-sided *t* test with the level of significance set at P<0.05.

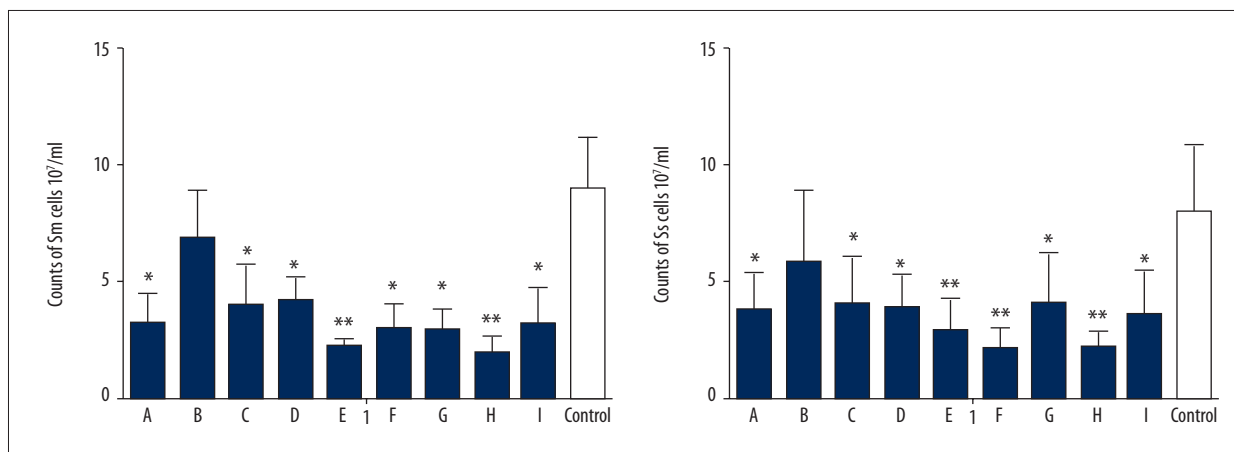
## Results

#### Inhibitory effect of supernatants on *P. gingivalis* growth and biofilm formation

The growth of *P. gingivalis* was significantly inhibited by *S. mutans* and *S. sanguinis* supernatants (P<0.05; Table 1). In the fluorescence images, the IOD of the supernatants of Sm and Ss showed a significant reduction as compared with controls, which indicated that the supernatants of Sm and Ss significantly inhibit the biofilm formation by *P. gingivalis* (P<0.05; Figure 1). The images of the biofilms captured by fluorescence biofilms are shown in Figure 2.



**Figure 2.** The effect of supernatants on Pg biofilm formation. Bacteria are stained with BaCLight Live/Dead staining kit: (A) *S. mutans*, (B) *S. sanguinis*, (C) control.



**Figure 3.** Viable Sm and Ss in the biofilms on different materials (A – nanoparticle resin composite Filtek Z350, B – Zinc phosphate cement, C – glass ionomer cement, D – Infiltrant resin, E – mineral trioxide aggregate (MTA), F – iRoot SP root canal sealer, G – iRoot BP root canal sealer, H – iRoot FS root canal sealer, I – AH Plus sealer). Data are expressed as the mean  $\pm$ SD, \*  $P < 0.05$ , \*\*  $P < 0.01$ .

### Adhesive ability of *S. mutans* and *S. sanguinis* on different dental materials

All groups except the zinc phosphate cement group (group B) exerted a strong inhibitory effect on the biofilm formation of *S. mutans* and *S. sanguinis* ( $P < 0.05$ ; Figure 3). In the SEM images, the bacterial number on the resin composite (group A), Glass ionomer cement (group C), Infiltrant resin (group D), Mineral Trioxide Aggregate (group E), iRoot SP root canal sealer (group F), iRoot BP root canal sealer (group G), iRoot FS root canal sealer (group H), and AH Plus sealer (group I) was significantly less than in the control group ( $P < 0.05$ ). The SEM images of the biofilm are presented in Figure 4.

### Discussion

Oral biofilms can be formed on a variety of oral hard tissue surfaces, which leads to the development and occurrence of caries and periodontal disease [17]. The formation of an acquired membrane on the surface of the repair materials promotes bacterial

adhesion to surfaces. *Streptococcus sanguis* is one of the main bacteria present in the human oral cavity, and *in vivo* studies have shown that *Streptococcus sanguis* has a negative relationship in dental plaque with bacterial pathogens suspected to be involved in periodontal disease [18]. Our experiments show that the supernatant of *Streptococcus sanguis* had a strong inhibitory effect on *P. gingivalis*, which was consistent with previous experimental results reported by Stinson et al. [19]. Previous studies have shown that *Streptococcus sanguis* can inhibit periodontal pathogens through the secretion of hydrogen peroxide and streptocin [20]. The bacteriostatic activity of the supernatant was observed in our experiments, which indicated that *Streptococcus sanguis* can produce antimicrobial substance [21]. The application of hydrogen peroxide has begun to make a real impact at the treatment of periodontal disease [22], so the use of *S. sanguis*, which is able to produce hydrogen peroxide, has become a new way to treat periodontal disease.

In our experiments, the glass ion cement biofilm had fewer bacteria, possibly because the 3M ESPE glass ion cement surface can release fluoride and change the pH, which could reduce the

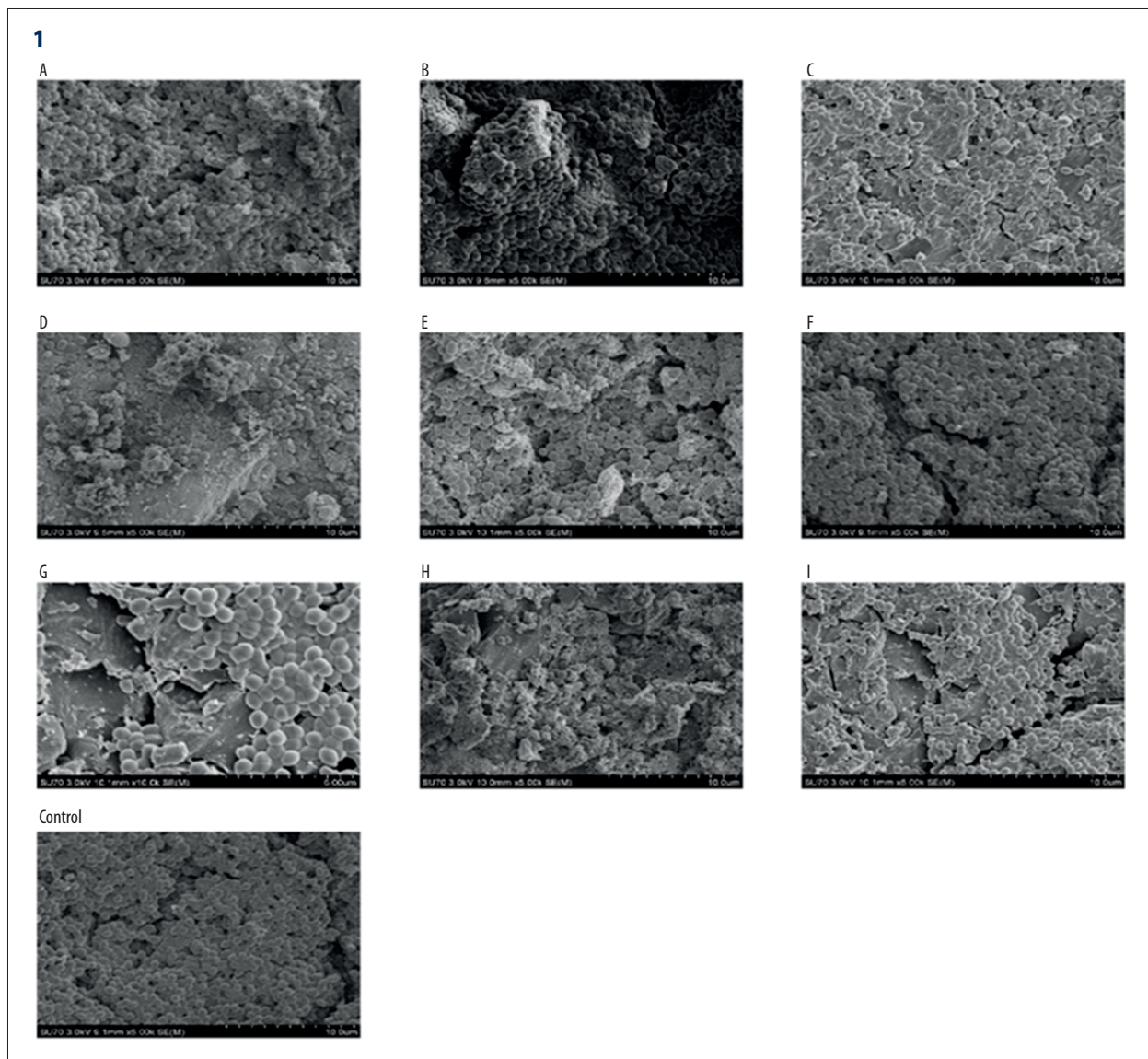


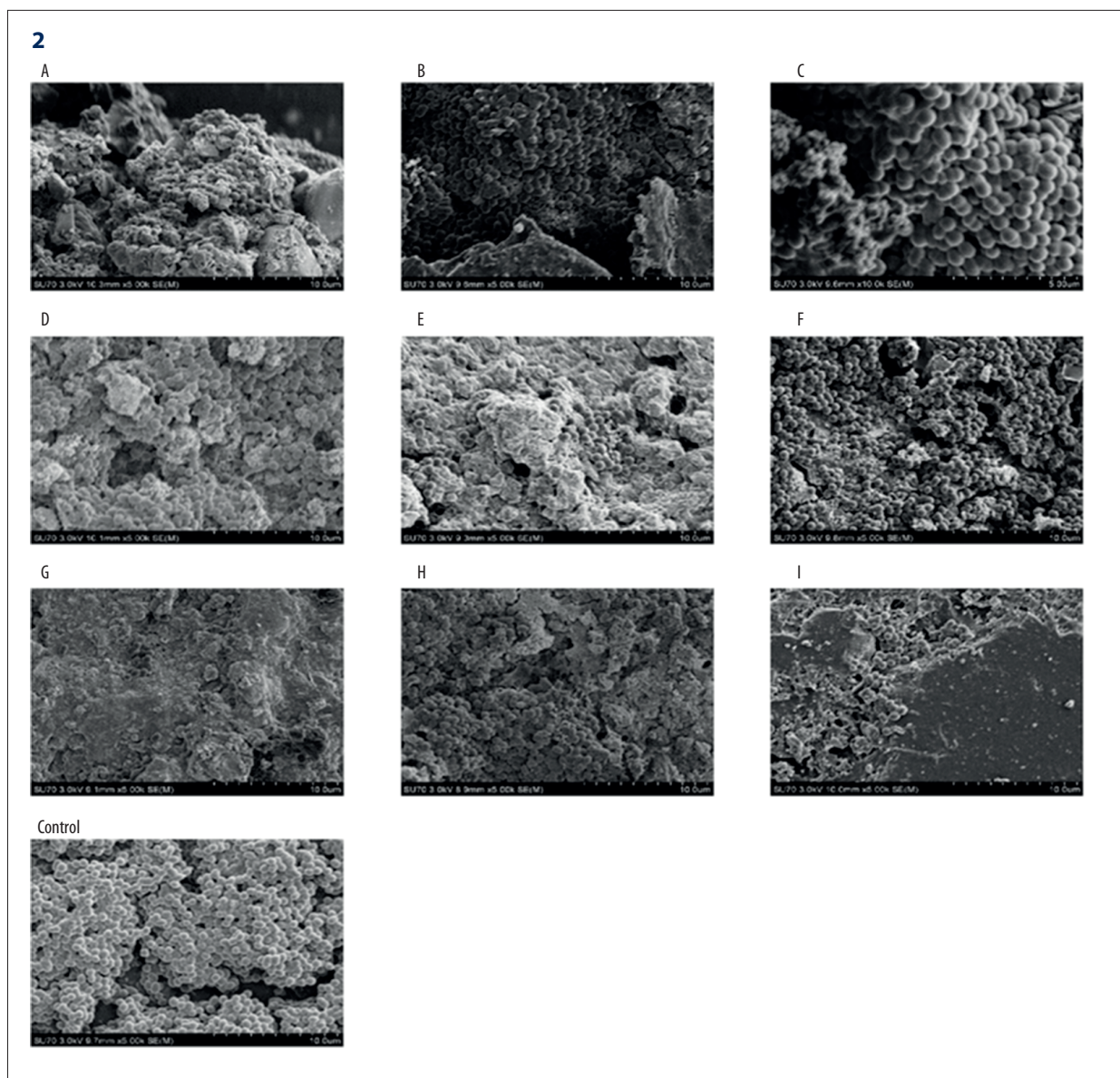
occurrence of caries [23,24]. Although composite resin materials lack effective antibacterial ingredients, to some extent bacterial adhesion can be reduced after polishing [25]. The main effects of zinc phosphate cement, MTA, and root canal sealer are to isolate irritation, rebase the cavity, and seal the root canals. Their surfaces are relatively coarse and lack effective antimicrobial properties, leading to an increased likelihood of bacteria forming biofilms on their surfaces [26].

There may be symbiotic relationships between many of the bacteria associated with caries and periodontal disease, but some bacteria may also inhibit each other. Studies have shown that the detection rate of periodontal pathogens such as *P. gingivalis* and *A. actinomycetem* can decrease when certain gram-positive cariogenic bacteria such as *S. mutans* and *S. sanguis* are also present [27]. *In vitro* studies of the relationship between periodontal

pathogens and caries have shown that *P. gingivalis* is antagonistic for *S. mutans*, which was consistent with our results [28].

Biofilm formation by bacteria on oral material is influenced by many factors, making the pathogenesis of periodontal pathogens and caries pathogenesis perplexing [29,30]. For example, during the early stages of periodontal disease, the etiology is closely associated with some inflammatory biomarkers [31,32]. However, in our experiment, the roughness and other physical parameters of the material surfaces were not evaluated [33] and the results do not show changes of inflammatory biomarkers *in vivo*; therefore, our results cannot be linked to specific features on the surface of the material and the effects *in vivo*. We plan to conduct further experiments after the comprehensive consideration of various parameters and to perform *in vivo* experiments in the future.





**Figure 4.** SEM images of the biofilm of *P. gingivalis* co-cultured with supernatants of *S. mutans* (1) and *S. sanguinis* (2). (A – nanoparticle resin composite Filtek Z350, B – zinc phosphate cement, C – glass ionomer cement, D – infiltrant resin, E – mineral trioxide aggregate (MTA), F – iRoot SP root canal sealer, G – iRoot BP root canal sealer, H – iRoot FS root canal sealer, I – AH Plus sealer).

## Conclusions

Our data indicate that the supernatants of *S. mutans* and *S. sanguinis* had significant inhibitory effects on the growth and biofilm formation of *P. gingivalis*, and the adhesive ability of

*S. mutans* and *S. sanguinis* are different on different dental materials. The results presented here will be of benefit to further studies related to dental caries, periodontal disease, and dental materials.



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