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In vitro cariogenic model of biofilm formation on selected dental materials – pilot study

Model *in vitro* tworzenia próchnicotwórczego biofilmu na wybranych materiałach stomatologicznych – doniesienie wstępne

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Keywords

composite, glass ionomer, *Streptococcus mutans*, *Actinomyces viscosus*, secondary caries

Summary

Introduction. Despite the progress made in restorative materials technology, secondary caries still remains a problem.
Aim. Present research aimed to investigate how the selected types of material and their roughness affected cariogenic biofilm formation. Nano-ceramic composite resin and glass-hybrid restorative system properties have been investigated.
Material and methods. Clinical strains of *Streptococcus mutans* and *Actinomyces viscosus* were isolated from plaque of children with caries and the cariogenic biofilm was formed on materials surface. In present study material roughness, total biofilm biomass, number of viable cells in biofilm and micromorphology was determined.
Results. Stronger biofilm generation has been shown to be associated with the type of dental restorative materials. A significant reduction was found in the *S. mutans* biofilm biomass formation and number of viable cells under the influence of application of glass ionomer compared to composite ($p < 0.05$) (mean ± SD: 0.114 ± 0.009 vs 0.076 ± 0.011 after 12 h; 0.138 ± 0.006 vs. 0.080 ± 0.005 after 72 h and mean ± SD: 1.27 ± 0.09 vs 1.32 ± 0.05 after 12 h; 1.28 ± 0.05 vs. 1.70 ± 0.07 after 72 h). A significant effect of the reduction of the number of *A. viscosus* viable microorganisms in the biofilm was identified on the glass ionomer material compared to the composite (mean ± SD: 1.10 ± 0.05 vs 1.20 ± 0.05 after 12 h; 1.11 ± 0.05 vs. 1.30 ± 0.05 after 72 h). The total biofilm biomass formed by *A. viscosus* on the composite material was lower compared to the controls (mean ± SD: 0.122 ± 0.011 vs. 0.132 ± 0.006 after 12 h; 0.132 ± 0.007 vs. 0.161 ± 0.007 after 72 h) but the optical density (OD) values of the created biofilm biomass indicate its more pronounced inhibitory effect on *A. viscosus* biofilm, as compared to the composite. Significant inhibitions of *A. viscosus* biofilm formation on glass ionomers have been ob-

served compared to the controls (mean ± SD: 0.122 ± 0.004 vs. 0.132 ± 0.006 after 12 h; 0.122 ± 0.005 vs. 0.161 ± 0.007 after 72 h).
Conclusions. Better anti-biofilm properties of glass ionomer compared with composite material can prove the advantage of glass ionomer materials in the prevention of secondary caries and in community children dental care.

Słowa kluczowe

kompozyt, glasjonomer, *Streptococcus mutans*, *Actinomyces viscosus*, próchnica wtórna

Streszczenie

Wstęp. Pomimo postępu w technologii materiałów odtwórczych próchnica wtórna nadal pozostaje problemem.
Cel pracy. Określenie wpływu badanego materiału wypełnieniowego i jego szorstkości na powstawanie próchnicotwórczego biofilmu. Zbadano właściwości nanoceramicznej żywicy kompozytowej i szklano-hybrydowego systemu odbudowy.
Materiał i metody. Ze szczepów *Streptococcus mutans* i *Actinomyces viscosus* wyizolowanych z płytki nazębnej dzieci chorych na próchnicę wytworzono biofilmy kariogenne na powierzchni materiałów wypełnieniowych. W niniejszym badaniu określono chropowatość materiału, całkowitą biomasę biofilmu, liczbę żywych komórek w biofilmie oraz mikromorfologię.
Wyniki. Wykazano, że silniejsze wytwarzanie biofilmu jest powiązane z rodzajem stomatologicznych materiałów odtwórczych. Stwierdzono istotne zmniejszenie tworzenia biomasy biofilmu *S. mutans* i liczby żywych komórek przy zastosowaniu glasjonomeru w porównaniu z kompozytem (p < 0,05) (średnia ± SD: 0,114 ± 0,009 vs 0,076 ± 0,011 po 12 godz.; 0,138 ± 0,006 vs 0,080 ± 0,005 po 72 godz. i średnia ± SD: 1,27 ± 0,09 vs 1,32 ± 0,05 po 12 godz.; 1,28 ± 0,05 vs 1,70 ± 0,07 po 72 godz.). Stwierdzono istotny wpływ zmniejszenia liczby żywych mikroorganizmów *A. viscosus* w biofilmie na materiale glasjonomerowym w porównaniu z kompozytem (średnia ± SD: 1,10 ± 0,05 vs 1,20 ± 0,05 po 12 godz.; 1,11 ± 0,05 vs. 1,30 ± 0,05 po 72 godz.). Całkowita biomasa biofilmu wytworzona przez *A. viscosus* na materiale kompozytowym była niższa w porównaniu z grupą kontrolną (średnia ± SD: 0,122 ± 0,011 vs. 0,132 ± 0,006 po 12 godz.; 0,132 ± 0,007 vs. 0,161 ± 0,007 po 72 godz.), ale wartości gęstości optycznej (OD) wytworzonej biomasy biofilmu wskazują na jej wyraźniejsze działanie hamujące na biofilm *A. viscosus* w porównaniu z kompozytem. Zaobserwowano istotne hamowanie tworzenia biofilmu *A. viscosus* na glasjonomerach w porównaniu z grupą kontrolną (średnia ± SD: 0,122 ± 0,004 vs. 0,132 ± 0,006 po 12 godz.; 0,122 ± 0,005 vs. 0,161 ± 0,007 po 72 godz.).
Wnioski. Lepsze właściwości przeciw biofilmowi glasjonomeru w porównaniu z materiałem kompozytowym mogą potwierdzać przewagę materiałów glasjonomerowych w profilaktyce próchnicy wtórnej oraz w publicznej opiece stomatologicznej dzieci.

Introduction

Secondary caries seems to be a serious problem in children, especially in the early childhood. Treatment of this process generates huge costs and untreated may lead to many complications, even severe, local and general in the child's organism. Dental materials become more and more modern and durable, but problem remains.

Contemporary dentistry places particular emphasis on caries prevention and minimally invasive treatment, saving hard tooth tissues, which should be the procedure of choice in each case of restorative treatment, using adhesive materials that meet strength and aesthetic requirements while maintaining proper indications for their use (1).

Each decision regarding the use of a specific restorative material must be individualized and be based on knowledge of the composition, physico-chemical properties and characteristics of the restorative material. When choosing the material, factors related to the severity of the carious lesion, the ability to control moisture during the procedure, oral hygiene and patient preferences should be taken into account. All this is aimed at reducing the development of secondary caries, or failure in the form of loss of all or part of the filling.

The problem of choosing restorative materials is also often associated with the economic possibilities of patients, and in a broader sense – with the economic model of organized healthcare in each country.

Composite materials, currently considered as basic restorative materials for reconstruction of anterior and posterior teeth, are recommended for permanent teeth fillings in all cavity classes (2). However, according to the results of the trials, currently the most commonly used materials for the reconstruction of cavities I and II class according to Black in deciduous molars are resin-modified glass-ionomer cements having at the same time remineralizing properties (3, 4). The lower popularity of fillings made of composite materials in primary dentition than in permanent dentition can be explained by the relatively complicated procedure of application of these materials, its multi-stage can be an important factor reducing the durability of fillings (5).

However, there are many doubts as to which materials are optimal for primary and permanent teeth. The insufficient number of randomized clinical trials available in the literature comparing various materials used to fill defects, especially in primary teeth, means that there is insufficient evidence to formulate clear clinical recommendations (6).

Nevertheless, secondary caries remains a big problem regardless of technological progress related to the quality of these materials (7, 8). The emerging dysbiosis between the cariogenic microorganisms species producing acids and the physiological flora of the host has an impact on their mechanical properties and is often the cause of microleaks of used dental materials, facilitating recurrent dental damage (9, 10). It is the main reason causing failure of restorations (11).

Streptococcus mutans is associated with the initiation of caries and is also isolated both from enamel carious lesions and hidden/closed dentinal lesions/dentin caries (12). *S. mutans* could be found in healthy and diseased sites and suggested that other species of *Lactobacillus*, *Prevotella*, *Propionibacterium*, nonmutans streptococci and *Actinomyces* also played important roles in caries initiation and biofilm community interactions (13-15).

Actinomyces viscosus, like *Streptococcus mutans*, is one of the most common supragingival plaque-forming cariogenic microorganisms (16-18). In addition to *Lactobacillus casei*, as strongly acidogenic and aciduric, it is also one of the two predominant deep caries microorganisms and one of the main etiological factors of secondary dental caries (19-22). Regulation of adhesive ability to the tooth surface of the above microorganisms can therefore be a good strategy for controlling pathogenic biofilms by affecting the relationship between the dental material used for the fillings and oral microflora, thus reducing or delaying the occurrence of secondary carious lesions in the teeth (23, 24).

Adhesive properties of the above microorganisms are the key features determining their pathogenicity, while their interactions with dental materials and the rest of the oral microflora may determine the activity of these features and the bacterial virulence under favourable conditions (9, 25, 26). Experimental data showed that the surface of the dental material, interaction of microorganisms and nutrient substrates needed for the metabolism of these bacteria determine the development or absence of cariogenic biofilm and secondary lesions.

Aim

The aim of the study was to assess and compare cariogenic biofilm formation on two dental materials in in vitro models with the use of clinical wild strains of *S. mutans* and *A. viscosus* isolated from children with caries.

Material and methods

Study groups

The study was performed in accordance with the Helsinki Declaration of 2013. Informing consent to the study procedure was obtained from all participants. The protocol was approved by the Bioethics Committee at the Jagiellonian University in Cracow (No. 1072.6120.183.2017).

The study was conducted between 2019 and 2021 and included 40 pediatric patients aged between 4-9 years old from Department of Pediatric Dentistry, Jagiellonian University Dental Clinic in Cracow, Poland. The study included bacterial strains isolated from dental plaque and saliva of patients diagnosed with caries. The results marked with reference numbers were entered onto a standardized examination chart. Examinations were conducted based on the criteria established by the World Health Organization for epidemiological studies (27).

The research group consisted of children with caries in dentin, defined as decay group 4-6 in the ICDAS II codes (International Caries Detection and Assessment System Coordinating Committee) (28).

The exclusion criteria were as follows: age below 4 or above 9 years, diabetes, periodontal disease, epithelial dysplasia, and inflammatory lesions of the oral mucosa. Antibiotics, non-steroid, anti-inflammatory medications, corticosteroids, and vitamin intake as well as professional fluoride prophylaxis within the last 3 months also resulted in a patient's exclusion from the study. Dental plaque was determined using the OHI-S index (Simplified Oral Hygiene Index) (29).

Preparation of the samples

A composite nano-ceramic filler Ceram X® (One Universal, Dentsply, DeTrey, Konstanz, Germany) and a glass-hybrid glass ionomer for bulk-fil-type fillings (EQUIA® Forte Fil, GC, Tokyo, Japan) were used in the study. These two materials were the most used from glass ionomers and composites group in the Department of Pediatric Dentistry for dental fillings in children. 40 samples were prepared according to the manufacturer's instructions for each of the materials. Ceram X® material samples with a thickness not exceeding 2 mm were irradiated with a 1200 mW/cm^2 polymerization diode lamp (GC D-Light Duo) for 20 seconds. Before preparing the samples from the glass ionomer, the capsule with the material was placed in a shaker and mixed for 10 sec. After the recommended setting time of 2.5 min, samples were covered with EQUIA® Forte Coat varnish and cured with light using a diode polymerization lamp for 20 sec (30).

The prepared materials were stored under lightproof and microaerophilic conditions prevailing in the mouth (laboratory incubator: 37°C, atmosphere enriched with 10% CO_2, > 95% relative humidity) for 24 h to allow complete polymerization of the composite and the glass ionomer. The slides were sequentially sterilized by the plasma sterilizer for one hour at 45°C to fix the resin surface.

In order to assess the homogeneity of the surface of the tested materials, their roughness was measured (SR, n = 10 of each material) using a needle profilometer (Bruker DektakXT, Germany). 10 randomly selected 1.75 mm fragments were measured in three-line scans for each material using a diamond tip with a radius of 2 µm and a tip angle of 90°. The cut-off level was set to 0.25. Data were expressed as Ra (µm).

The materials fixed on the slides were then transferred to the sterile microtiter plates containing 2 ml sterile PBS (Phosphate Buffered Saline) in each well and stored at a room temperature for another 10 days to allow unreacted monomers to leach from the slides coated with dental materials.

Saliva and dental plaque collecting

Saliva samples were collected from all participants according to the protocol proposed by Szczeklik et al., 2 hours after morning tooth brushing with fluoride toothpaste (31). The participating subjects were fasting before saliva collection. Participants rinsed they mouth with deionised water for 30 sec. and expectorated oral contents. The stimulated saliva was collected by chewing cotton swab for 3 min. Soaked swabs were placed in salivettes which were immediately centrifuged for 2 min at 4°C and 1000 rpm. Collected material was stablished to sterile 1.5 ml Eppendorf type tubes which were frozen at -80°C until assayed. Dental plaque from all tooth surfaces was collected using sterile dental brush according to the protocol proposed by Krzyściak et al. (32).

Microbiological analysis

The samples were transported within 4 hours to the laboratory in 1 ml sterile physiological saline, pH 7.0 (PBS) at room temperature. The samples were disrupted by gentle vortexing and sonication for 30 sec. Subsequently, serial dilutions of the starting solution were prepared in sterile physiological saline. Samples with the above dilutions were inoculated on plates with 10% blood agar and on selective media used to detect certain microorganism groups described by Krzyściak et al. (32).

The inoculated media were incubated in microaerophilic conditions in the presence of 5% CO_2 at a temperature of 37°C over a period of 24-48 hours. Based on their morphology, grown on selective media were counted.

The bacteria species obtained in the tests were verified by means of mass spectrometry (MS) system MALDI with TOF (Bruker Daltonik, Germany). The identification of bacteria species was possible by comparison of obtained peptides molecular weight, charge and time-of-flight distribution spectra with reference spectra from database (MALDI Biotyper 3.0 software). The likehood of correct identification was expressed as point indicator. The value of that indicator for reliable identification in this study was stablished as 2.000 or greater.

Biofilm model

Microbiological tests with 40 clinical isolated *S. mutans* and *A. viscosus* strains were carried out in 24-well sterile flat-bottomed microtiter plates with the covered polystyrene slides of a 12 mm diameter placed on the bottom.

Prepared samples of composite materials and glass ionomer were placed in 2.0 ml of BHI culture medium with 5% sucrose containing bacterial strains and incubated for 72 h at 37°C in microaerophilic conditions in a 10% CO_2 atmosphere. Inoculation of the medium with a suspension of pure cultures was carried out in a logarithmic phase of bacterial growth at a concentration of 1×10^7 cells/ml.

The control samples (polystyrene disks without dental material) contained bacterial suspensions of *S. mutans* and *A. viscosus* reference strains: ATCC 25175 and ATCC 43146 in 0.9% NaCl which were incubated under the same conditions as the test samples.

In vitro biofilm tests

The ability of biofilm formation on the selected dental materials was assessed using a closed model based on a microtiter plate. The formed biofilm biomass was measured by crystal violet (CV) staining according to the method described in our previous studies (33).

100 µl of the standardized suspension of *S. mutans* and *A. viscosus* was transferred to each well of a 96-well microtiter plate coated with the tested dental materials, which was then incubated for 90 min at 37°C to initiate an adhesion of the tested bacterial strains. The suspension was aspirated and each well was washed three times with PBS to remove planktonic cells. 100 µl of fetal bovine serum was consequently added to each well and incubated under the same conditions for 8 h. The wells were washed three times with PBS and 100 µl of a standardized bacterial suspension was added to each well. Control samples contained 100 µl PBS (PBS control) or 100 ml BHI broth with 5% sucrose. The medium was changed once and the plates were incubated for 72 h.

Biofilm generation by CFU/ml determination

To determine the number of bacteria in the biofilm, the cultures were performed after thoroughly rinsing the plates with sterile PBS, scraping the biofilm from the bottom of the wells and suspending it in 100 µl sterile saline. Such a bacterial suspension was serially diluted and inoculated on BHI agar media with 5% sheep blood. The number of grown colonies was counted after 24, 48 and 72 h of overnight culture and expressed as CFU/ml according to the formula: CFU/ml = (number of colonies on the plate × 10) ÷ dilution.

Analysis of biofilm formation by determining its total biomass

Biofilm biomass was assessed after 24, 48 and 72 h of incubation by staining with CV. The biofilm was dipped in 99% methanol for 20 min and then air dried. 125 µl of 0.1% CV solution was added to the wells which were washed three times with PBS after 20 min. The plates were dried by shaking after each washing step. After the final washing step, the samples were allowed to dry completely. Finally, the bound CV was released by adding 200 µl of 95% ethanol followed by incubation for 15 min at a room temperature. The contents of the wells were mixed by repeated pipetting, and then 125 µl of the suspension was transferred to the wells of a clean 96-well flat-bottom microtiter plate. Absorbance was measured at a wavelength of 540 nm. All steps were carried out at a room temperature. The study was conducted in two independent experiments, separately for *S. mutans* and *A. viscosus*. The biofilm formation curve was plotted based on the obtained data.

Analysis of biofilm formation using scanning electron microscopy (SEM)

Discs (Agar Scientific, UK) of a 13 mm diameter previously coated with dental materials were placed in the wells of a 24-well sterile culture plate to form a biofilm according to the procedure described above. After 24, 48 and 72 h, the samples were fixed in 1 ml of 2.5% glutaraldehyde solution for 1 h, dehydrated in a series of graduated ethanol for 20 min each, followed by immersion in 100% alcohol for 1 h. The discs were dried in an incubator under microaerophilic conditions for 24 hours. After drying, the samples were transferred to aluminium slides and sprayed with gold (160 s, 40 mA). The samples were examined and photographed under a JEOL JSM-35CF (SEM) 15 kV scanning electron microscope. These experiments were performed at each time point on two monospecies biofilm cultures of *S. mutans* and *A. viscosus*. Figure 1 illustrates conducted experimental procedure process.

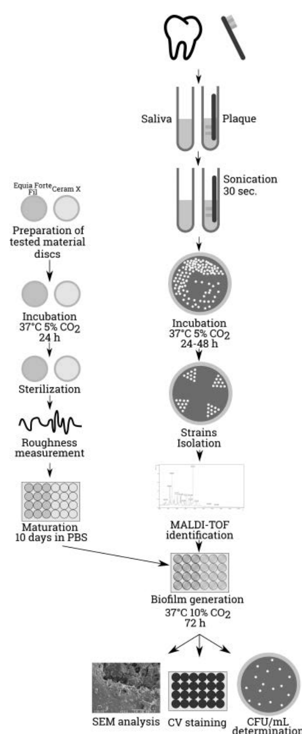


Fig. 1. Scheme illustrates the protocol applied in the experiment

Statistical analysis

Data were analyzed using R environment (34) with significance level set at $p \leq 0.05$. Material roughness, biofilm biomass and biofilm generation were analyzed using ANOVA. Due to the fulfilled assumptions about the normality of distribution and homogeneity of variance in the groups, the post-hoc Tukey test was used for all data sets to compare the variables between each other ($\alpha = 0.05$). Graphs were generated using ggplot2 package (35).

Results

Material roughness of the tested materials

The results regarding the surface of the examined dental materials are presented in table 1. The roughness of resin-based composites (RBC) was significantly lower compared to the glass ionomer, what affects the ability to form biofilm ($p \leq 0.01$).

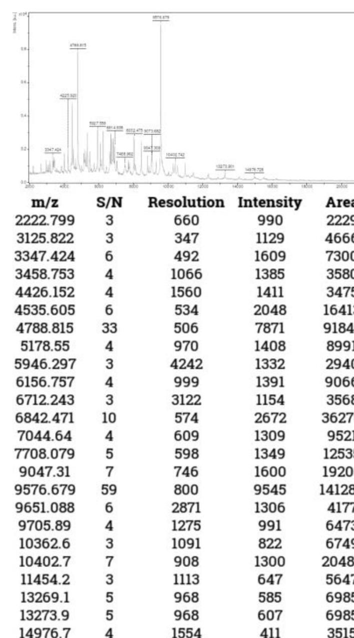
Tab. 1. Analysis of the surface roughness of dental materials after exposure to UV in the presence of different media: water, collective saliva, and broth heart infusion medium supplemented with 5% sucrose. Mean +/- SD are presented; different letters in superscript indicate significant differences between the groups (Schefe Test).

Material	Medium	Ra [µm]
Composite	Water	0.268 (0.023)$^{a, b}$
	BHI	0.341 (0.031)^{a}
	Saliva	0.296 (0.024)^{a}
Glass ionomer	Water	0.872 (0.031)^{b}
	BHI	1.321 (0.028)^{c}
	Saliva	0.963 (0.031)^{a}

Note: The same letter in superscript in line means that there is no significant differences between marked groups. Different letters mean that there is significant difference between groups ($p < 0.05$)

Microbiological identification

Identification results of bacterial strains isolated from the dental plaque of children with caries was carried out using the MALDI-TOF MS technique. Comparing the pattern with database MALDI Biotyper 3.0 allowed the identify bacteria species (fig. 2).



m/z	S/N	Resolution	Intensity	Area
2222.799	3	660	990	2229
3125.822	3	347	1129	4666
3347.424	6	492	1609	7300
3458.753	4	1066	1385	3580
4426.152	4	1560	1411	3475
4535.605	6	534	2048	16413
4788.815	33	506	7871	91841
5178.55	4	970	1408	8991
5946.297	3	4242	1332	2940
6156.757	4	999	1391	9066
6712.243	3	3122	1154	3568
6842.471	10	574	2672	36270
7044.64	4	609	1309	9521
7708.079	5	598	1349	12535
9047.31	7	746	1600	19206
9576.679	59	800	9545	141284
9651.088	6	2871	1306	4177
9705.89	4	1275	991	6473
10362.6	3	1091	822	6749
10402.7	7	908	1300	20488
11454.2	3	1113	647	5647
13269.1	5	968	585	6985
13273.9	5	968	607	6985
14976.7	4	1554	411	3515

Fig. 2. Sample result of bacterial strain analysis obtained with MALDI-TOF MS. Comparing the pattern with database MALDI Biotyper 3.0 allowed the identify bacteria species

Biofilm formation (evaluation of biomass)

The results of mono-species biofilm formation (biomass, the number of biofilm forming colonies, structure and morphology) of *S. mutans* and *A. viscosus* were analyzed for 40 samples of dental materials, i.e. glass ionomer and composite. The presented studies showed the existence of statistically significant differences ($p < 0.0001$, ANOVA) both between the biomass of formed biofilms and the number of microorganisms forming them on the tested materials (both on composites and glass ionomers). A statistically significant decrease in biofilm formation (both biomass and the number of biofilm colonies) was observed at each time point (after 12, 24, 48, and 72 h of the culture) for both *S. mutans* and *A. viscosus*. The results are presented in figures 3 and 4.

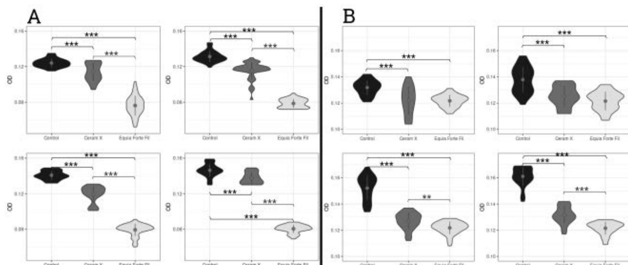


Fig. 3a, b. Violin plot of *S. mutans* (a) and *A. viscosus* (b) total biofilm mass formed on the surfaces of the tested materials and the control surface after 12, 24, 48, and 72 h of culture in BHI broth enriched with 5% sucrose. Black clamps indicate the significance of the differences (Tukey's test) relative to the control surface, blue clamps indicate the differences between the tested materials; the number of stars means the value of p (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). The red dots indicate the median and the red lines represent the quartile range while shape represents distribution of data

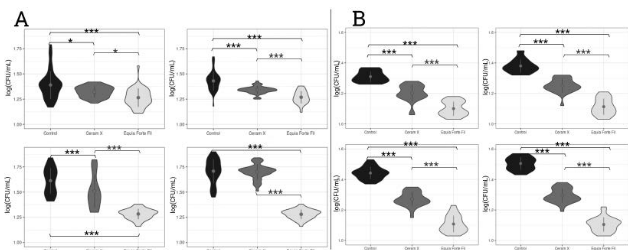


Fig. 4a, b. Violin plot of total number of colonies of *S. mutans* (a) and *A. viscosus* (b) formed on the surfaces of the tested materials and the control surface after 12, 24, 48, and 72 hours of culture in BHI broth enriched with 5% sucrose. Black clamps indicate the significance of the differences (Tukey's test) relative to the control surface, blue clamps indicate the differences between the tested materials; the number of stars means the value of p (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). The red dots indicate the median and the red lines represent the quartile range while shape represents distribution of data

Measurement of the total biomass expressed as OD (optical density)

The total mass of the biofilm formed by *S. mutans* on the composite material was lower compared to the control at the particular time points (mean ± SD: 0.114 ± 0.009 vs. 0.124 ± 0.005 after 12 h; 0.115 ± 0.009 vs. 0.131 ± 0.006 after 24 h; 0.119 ± 0.010 vs. 0.141 ± 0.004 after 48 h; 0.138 ± 0.006 vs. 0.146 ± 0.007 after 72 h). Moreover, a significant higher effect (as compared to composite) of inhibition of biofilm generation was identified on the glass ionomer material (mean ± SD: 0.114 ± 0.009 vs 0.076 ± 0.011 after 12 h; 0.115 ± 0.009 vs 0.079 ± 0.005 after 24 h; 0.119 ± 0.010 vs. 0.079 ± 0.006 after 48 h; 0.138 ± 0.006 vs. 0.080 ± 0.005 after 72 h). Graphic interpretation of the observed differences is shown in figure 3a.

The total biofilm biomass formed by *A. viscosus* on the composite material was lower compared to the controls after 12, 24, 48, and 72 hours of incubation (mean ± SD: 0.122 ± 0.011 vs. 0.132 ± 0.006 after 12 h; 0.125 ± 0.008 vs. 0.138 ± 0.010 after 24 h; 0.127 ± 0.006 vs. 0.152 ± 0.009 after 48 h; 0.132 ± 0.007 vs. 0.161 ± 0.007 after 72 h).

In the case of glass ionomer material, the OD values of the created biofilm biomass indicate its more pronounced inhibitory effect on *A. viscosus* biofilm, as compared to the composite. Since the beginning of the experiment, significant inhibitions of *A. viscosus* biofilm formation on glass ionomers have been observed compared to the controls (starting from 12 hours of incubation, mean ± SD: 0.122 ± 0.004 vs. 0.132 ± 0.006 after 12 h; 0.122 ± 0.007 vs. 0.138 ± 0.010 after 24 h; 0.122 ± 0.005 vs. 0.152 ± 0.009 after 48 h; 0.122 ± 0.005 vs. 0.161 ± 0.007 after 72 h). The described effects together with statistically significant differences are presented in figure 3b.

Determination of the number of live microorganisms in the biofilm

The number of viable microorganisms in the biofilm formed by *S. mutans* on the composite material was lower compared to the control at the particular time points (mean ± SD: 1.32 ± 0.05 vs. 1.39 ± 0.14 after 12 h; 1.34 ± 0.04 vs. 1.43 ± 0.09 after 24 h; 1.51 ± 0.16 vs. 1.61 ± 0.13 after 48 h). No statistically significant differences were identified at the last time point (1.70 ± 0.07 vs. 1.71 ± 0.12 after 72 h). Moreover, a more significant effect of the reduction of viable microorganisms in the biofilm was identified on the glass ionomer material compared to the composite (mean ± SD: 1.27 ± 0.09 vs 1.32 ± 0.05 after 12 h; 1.27 ± 0.06 vs 1.34 ± 0.04 after 24 h; 1.28 ± 0.05 vs 1.51 ± 0.16 after 48 h; 1.28 ± 0.05 vs. 1.70 ± 0.07 after 72 h). Graphic interpretation of the observed differences is shown in figure 4a.

The number of viable microorganisms in the biofilm formed by *A. viscosus* on the composite material was lower compared to the control (polystyrene disk) (mean ± SD: 1.20 ± 0.05 vs. 1.31 ± 0.04 after 12 h; 1.25 ± 0.05 vs. 1.38 ± 0.04 after 24 h; 1.27 ± 0.15 vs. 1.44 ± 0.14 after 48 h; 1.30 ± 0.05 vs. 1.50 ± 0.04 after 72 h). A more significant effect of the reduction of the number of viable microorganisms in the biofilm was identified on the glass ionomer material compared to the composite, too (mean ± SD: 1.10 ± 0.05 vs 1.20 ± 0.05

after 12 h; 1.11 ± 0.05 vs 1.25 ± 0.05 after 24 h; 1.11 ± 0.05 vs 1.27 ± 0.05 after 48 h; 1.11 ± 0.05 vs. 1.30 ± 0.05 after 72 h). These data are presented in figure 4b.

Micromorphological characteristics of biofilms

A stronger inhibitory effect on the *S. mutans* and *A. viscosus* strains used was observed in the case of glass ionomer materials compared to composites, as illustrated in the images from the scanning electron microscope (fig. 5, 6). The examples of micromorphology of biofilms created on composite material are presented in figure 5a-d (*S. mutans*) and figure 5e (*A. viscosus*), while biofilms created on glass ionomer are shown in figure 6a-f (*S. mutans*) and figure 6g (*A. viscosus*).

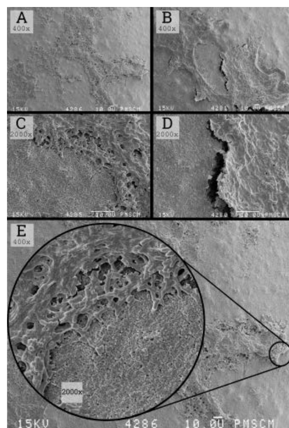


Fig. 5a-e. Scanning electron microscopy. Biofilm formed by *S. mutans* on Ceram X® (nano-ceramic composite resin) after 24 h of biofilm formation at 37°C, 10% CO_2. Magnification: 400x (a, b) and 2000x (c, d). Biofilm formed by *A. viscosus* on Ceram X® (nano-ceramic composite resin) after 24 h of biofilm formation at 37°C, 10% CO_2 (e). Magnification: 400x and 2000x

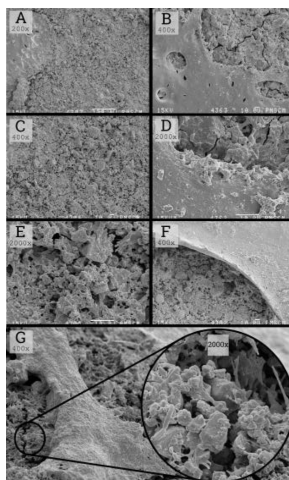


Fig. 6a-g. Scanning electron microscopy. Biofilm formed by *S. mutans* on Equia® Forte Fil after 24 h of biofilm formation at 37°C, 10% CO_2. Magnification: 200x (a), 400x (b, c) and 2000x (d, e). Biofilm formed by *A. viscosus* on Equia® Forte Fil after 24 h of biofilm formation at 37°C, 10% CO_2 (e). Magnification: 400x and 2000x (f, g)

Discussion

Two species of microorganisms, i.e. *S. mutans* and *A. viscosus*, were selected for the presented study due to their key role in the etiology of dental caries. *S. mutans* is considered as one of the most important factors in the development and initiation of primary caries. In addition, its esterases as well as salivary and leukocyte esterases significantly reduce the mechanical properties of composite fillings (36). This process may lead to the formation of micro-fractures and the development of secondary caries, which is among others caused by *A. viscosus* (37, 38).

The present study used composite materials with nanoceramic filler (One Universal, Dentsply, DeTrey, Konstanz, Germany) and glass-hybrid glass ionomer for bulk-fil fillings (EQUIA® Forte Fil, GC, Tokyo, Japan). Composite is a dimethacrylate resin containing modified nanoceramic filler particles. According to the manufacturer's information, it shows a limited release of free monomers, nevertheless, the samples were incubated for 10 days in PBS to remove possible monomer residues. This action was intended to prevent the occurrence of a potential effect of microbial growth or inhibition by some free monomers observed by Lin et al. (39). Examined glass ionomer filling system contains glass filler and ultra-fine highly reactive glass particles, which accelerate and strengthen the formation of the hybrid matrix upon mixing. Thanks to the innovative glass hybrid technology, the availability of ions is increased, and the matrix is characterized by improved physical properties, higher abrasion resistance and high fluoride ion release.

The present study used a simplified in vitro model of a single-species biofilm. It is worth emphasizing that the monospecies biofilm model is a great simplification of the conditions prevailing in vivo, where processes between different microbial species play an important role. A limitation of this model may also be the use of a glass disk as a control surface, which is significantly different from the tooth enamel. In vivo models involving patients, however, are more complex but completely reflecting the physiological environment, nevertheless, they demonstrate many variable factors such as microflora composition, diet, oral health, ph cycling, etc. (36-38). These factors may increase the variability of the assessed parameter in groups, making the analysis of the basic mechanisms difficult. The in vitro model, despite the presented limitations, was used due to the easy reproducibility of the conditions and the ability to assess the basic mechanisms.

This observational study concerned the assessment of the impact of dental composites and glass ionomers, commonly used in filling of the carious defects, on the ability to generate a mono-species biofilm by *S. mutans* and *A. viscosus* in vitro. A noticeable inhibition of biofilm formation (both biomass and the number of bacterial colonies in the biofilm) was observed when using the glass ionomer in comparison to the composite. This is consistent with the in vitro results of other groups of the researchers (40-43), who noticed

significant differences in the ability to form a biofilm and the viability of microorganisms on the surfaces of composite or glass ionomer materials compared to the control surface. It should be noted that the anti-biofilm activity of glass-ionomer materials differs significantly between the products of particular manufacturers, which is related to the kinetics of fluoride ion release, which has been described by Hahnel et al. (40). In addition, as in the present study, the researchers showed a relationship between the parameters of the surface of materials and its anti-biofilm properties, but only in the initial phase of biofilm formation (40). It should be noted that the surfaces of the fillings may change their physical in vivo properties over time, as demonstrated by Barbosa et al. (44). In this experiment, the authors showed a loss of hardness of glass ionomer and composite fillings over time which was correlated with the amount of the formed biofilm (44). This fact proves that proper oral hygiene is one of the key factors in preventing secondary caries.

Significant material-related interactions related to inhibitory effects were found. Glass ionomers showed stronger inhibitory properties on both tested strains, affecting the reduction of biomass and the number of microorganisms in the biofilm. The observed effect in the case of glass ionomer material persisted throughout the experiment and both measured parameters did not increase significantly. The reason for this observation may be the cariostatic and antibacterial effect of glass ionomer cements due to the release of fluoride ions by the glass ionomer, which are known for their antimicrobial properties associated with inhibition of the bacterial glycolysis process (45, 46). In addition, fluoride ions in vivo enhance the chemical resistance of dental hard tissues to organic acids, limit demineralization and strengthen the remineralization of the tissues (47, 48). All of the above results consistently point to better antibacterial properties of glass ionomers compared to composites regarding the bacteria considered to be pioneer colonizers, i.e. *S. mutans* or *A. viscosus*, which produce acids and are recognized as the main risk factor for demineralization and the development of carious lesions in humans (49).

The conducted studies found that the amount and composition of the bacterial plaque on the dental materials is different and depends on the chemical composition and roughness of their surface. For this reason, manufacturers add substances to fillings that can directly affect the reduction of biofilm growth and increase the resistance of hard tooth tissues. An example of such materials may be glass ionomer cements containing fluorine ions, which are released into the oral environment without adversely affecting the integrity and physicochemical parameters of the dental materials. In addition, glass ionomer cements can absorb fluoride from the oral environment, acting as a reservoir which releases the ions into saliva in the event of a pH decrease, causing long-term cariostatic effect and inhibiting bacterial growth (50). It is possible to obtain antibacterial activity of composite materials and binding systems by using two types of chemical substances: those releasing and those not releasing antibacterial compounds. The first group of composite materials that release an antimicrobial substance contains, among others, chlorhexidine digluconate and chlorhexidine acetate, fluorine compounds, amorphous calcium phosphate (ACP) and quaternary ammonium dimethacrylate (QADM), which are released from the surface of the material for several days after polymerization of the composite. It is believed that they exhibit antibacterial activity over a larger area than the filling, but their addition negatively affects the mechanical properties and adhesion of the materials whose structure becomes porous. The second group of the materials are composites that do not release the antibacterial substance, whose effect is initiated during the contact with the microorganism. This group includes 12-methacryloyoxy dodecyl pyridinium bromide (IVDBA), methacryloxylethyl cetyl ammonium chloride (DMAE-CB), cetylpyridine chloride (CPC), quaternary ammonium polyethylenimine salt (QPE), 2-dimethyl-2-dodecyl-1-methacryloryetammonium iodine (DDMA) and furanone derivatives (51, 52).

Equia® Forte Coat varnish, which contains a monodisperse nanofiller creating a stable, smooth and tight protective coating on the surface of the glass ionomer filling, is an important factor affecting the reduction of roughness and increasing the strength of the Equia® Forte Fil material (53).

Controlling and reducing the adhesion of microorganisms to fillings is necessary to prevent the formation of biofilm on their surface. This strategy allows to extend the time of use of the filling in the mouth and prevent the development of secondary caries. It should be emphasized that adhesion control consists not only of factors such as material composition, filler particle size, surface charge and surface roughness, but also dietary and hygienic habits of the patient and the use of anti-caries prophylaxis (16).

The more favourable properties of the glass ionomer in terms of lower microorganism concentration and release of fluoride ions disturbing the metabolism of bacteria, is an indication for the use of this material in patients with a high risk of caries and a large amount of caries lesions in the first phase of treatment in order to quickly stop the progression of the disease.

It was shown that the tested materials significantly inhibit the growth of both tested species, expressed as a decrease in biofilm mass as well as a decrease in the total number of live microorganisms. What's more, glass-hybrid ionomer (EQUIA® Forte Fill), despite a greater surface roughness, has better anti-biofilm properties in relation to *S. mutans* and *A. viscosus*, than the composite material with Ceram X® nanoceramic filler. This effect should be associated with the release of fluoride ions by this material, which, combined with the fact that it exhibits a high degree of adhesion to enamel and dentin, and favorable coefficient of thermal expansion, can prove the advantage of glass ionomer materials over composite materials in the prevention of secondary

caries, and as a result improve the oral health of children and lower dental healthcare costs.

This material is also characterized by improved mechanical properties, compared to other glass-ionomers, such as strength or abrasion resistance, making it extremely useful in the final filling of carious defects, especially in children with high caries activity (54). Another important application of glass-ionomers is interim therapeutic restorations (ITR) in patients with multiple foci of active caries to stabilize the disease, limit its further spread in the mouth, and prevent possible inflammatory complications from the pulp. It is often the method used in patients requiring special care and in very young, non-cooperating children, in whom final restorations of composite materials cannot be made.

The most important factors that should be taken into account when choosing the most suitable filling material are: ease of donning, physical and chemical properties (durability of the filling), but also remineralizing effects (55). In the case of pediatric dentistry, the age of the child is also equally important, which is directly related to the child's cooperation (the possibility of isolating the operating field from moisture and performing local anesthesia, duration of the procedure). The child's age also indicates the time remaining until the primary tooth exfoliates, and thus how long the filling should remain in the mouth. The level of caries risk is extremely important. In children at high risk of this disease, filling carious cavities is primarily aimed at reducing infection and bacterial numbers, not just aesthetic and permanent reconstruction as in children at low risk of caries (54).

The problem of choosing restorative materials is often associated with the economic possibilities of patients, and more broadly with the economic possibilities of organized healthcare in a given country. In financial terms, the risk of secondary caries and the consequent need for subsequent replacement of fillings, as well as the cost of the restorative material and accessories necessary for its application (e.g. cofferdam in the case of composites), is becoming increasingly important. In this approach, glass-hybrid ionomers come to the fore. This study is a pilot study, there is a need to extend it with more types of composites and glass ionomers. Compomers are commonly used materials in pediatric dentistry too and should be included into the study.

Conclusions

Our study revealed an advantage of glass ionomer material over composite material in lower the cariogenic biofilm formation, so also in the prevention of secondary caries, and as a result improve the oral health of children and lower dental healthcare costs.

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Konflikt interesów

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