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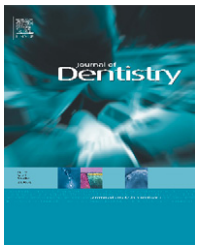

An *in vitro* quantitative antibacterial analysis of amalgam and composite resins

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ABSTRACT

Objectives: Antibacterial properties of restorative dental materials such as amalgam and composite resins may improve the restorative treatment outcome. This study evaluates the antibacterial properties of three composite resins: Z250, Tetric Ceram, P60 and a dental amalgam *in vitro*.

Methods: *Streptococcus mutans* and *Actinomyces viscosus* served as test microorganisms. Three quantitative microtiter spectrophotometric assays were used to evaluate the effect of the restorative materials on: (i) early-stage biofilm using a direct contact test (DCT); (ii) planktonic bacterial growth; (iii) bacterial growth in the materials' elute. For comparison purposes, agar diffusion test (ADT) was also performed.

Results: The effect of the composite resins on bacterial growth was minimal and limited to a few days only. One-week-aged composites promoted growth of *S. mutans* and *A. viscosus*. The antibacterial properties in direct contact were more potent than in planktonic bacterial growth. Amalgam showed complete inhibition of both bacteria in all phases, and the effect lasted for at least 1 week. The materials' elute had no effect on both bacterial growth with the exception of complete inhibition of *S. mutans* in amalgam. The later results correlated with the ADT.

Conclusions: The present findings demonstrate potent and lasting antibacterial properties of amalgam, which are lacking in composite resins. This may explain the clinical observation of biofilm accumulated more on composites compared to amalgams. It follows that the assessment of antibacterial properties of poorly-soluble materials has to employ more than one assay.

1. Introduction

The interaction between bacteria and solid surfaces often results in the attachment and subsequent proliferation of the microorganisms, culminating in a well-defined biofilm.[1,2] Bacteria in biofilm are organized in a three-dimensional structure enclosed within an extracellular matrix derived both from the bacteria and from the environment.[1,2] Cells growing in biofilm display hyper-resistance to antibacterial agents which, is attributed in part to the spatial structure, and in part

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to the alteration of a number of genes expressed in response to the proximity of a specific surface.[3–6]

The principal etiologic agents that initiate and propagate dental diseases are permanent habitants of the biofilm formed on teeth and restorations. Restorative materials with long-lasting antibacterial surface properties may reduce the biofilm and thus disease recurrence.

Previous studies have demonstrated that composite resin restorations accumulate biofilm more than amalgam restorations,[7] and this *in vivo* finding may be attributed to the antibacterial properties.

The antibacterial behavior of many dental materials was examined using the agar diffusion test (ADT)[8,9] or by testing the minimum inhibitory concentration (MIC).[9–14] These methods are based on measuring water-soluble components released from the bulk of the materials and are often used to evaluate antibiotics. The suitability of these methods for testing restorative materials, intended to last in an aqueous environment for many years is questionable.[15]

In the present study a direct contact test was used to quantify the bacteria in both early stage biofilm and in planktonic environments formed on restorative materials. The results were compared with those obtained using the ADT.

2. Materials and methods

2.1. Bacteria and growth conditions

Streptococcus mutans (ATCC#27351) and *Actinomyces viscosus* (ATCC#43146), originally isolated from human dental plaque, were used in this study. Bacteria were cultured aerobically overnight at 37 °C in 5 ml of brain–heart infusion broth (BHI) (Difco, Detroit, MI). To avoid the formation of long streptococcal chains or large *actinomyces* aggregates, the top 4 ml of the undisturbed overnight bacterial cultures were transferred to a new test tube and centrifuged for 10 min at 3175 × *g*. The supernatant was discarded and the bacteria were re-suspended in 5 ml phosphate-buffered saline (PBS), pH 7.5 (Sigma, St. Louis, MO) and mixed gently by vortex for 10 s. The suspensions were adjusted to an optical density (OD) of 0.5 at 650 nm, 10-fold serially diluted and plated on BHI agar to determine colony-forming units (CFU).

In order to minimize contamination 0.0625 g/ml of bacitracin (Sigma, St. Louis, MO) was added to the *S. mutans* growth medium and to the PBS. In experiments performed with *A. viscosus* lack of contamination was verified by microscopic examination.

2.2. Restorative materials tested

The antibacterial properties of four restorative materials were tested: (i) Z250 a composite resin with a filler of 60% zirconia/silica, average particle size 0.01–3.5 μm, and a matrix of BIS-GMA, UDMA, BIS-EMA (3M ESPE, Dental St. Paul, MN, USA), (ii) Tetric Ceram a composite resin with a filler of 60% barium glass, ytterbium trifluoride, Ba–Al–fluorosilicate glass, highly dispersed silicon dioxide, spheroid mixed oxide, average particle size 0.04–3.0 μm, BIS-GMA, UDMA TEGDMA matrix (Ivoclar Vivadent Ltd., Auckland, New Zealand), (iii) P60 a composite resin with a filler of 61% zirconia/silica average particle size 0.19–3.3 μm, BIS-GMA, UDMA, BIS-EMA (3M ESPE Dental St. Paul, MN), and (iv) F-400 micrograin lathe cut dental amalgam (70% Ag, 26% Sn, 3.5% Cu, 0.5% Zn; SDI Victoria, Australia).

2.3. Kinetic measurements of bacterial growth

2.3.1. Direct contact test—early stage biofilm

The experimental setup is shown in Fig. 1. A 96-well flat bottom microtiter plate (Nunclon, Nonc, Copenhagen, Denmark) was vertically positioned. Seven wells were coated with tested material samples; 30 ± 4 mg of composite samples and 45 ± 5 mg of the amalgam samples. The samples were applied on the sidewall using a flat-ended dental instrument (spatula) ensuring uniform surface area. The composite resin samples were polymerized according to the manufacturers' instructions. Amalgam samples were allowed to set for 30 min before testing.

To form an early biofilm on the tested material, 10 μl of the bacterial suspension (10^6 CFU) was placed on the sample in a set of seven wells, and the plate was incubated in a vertical

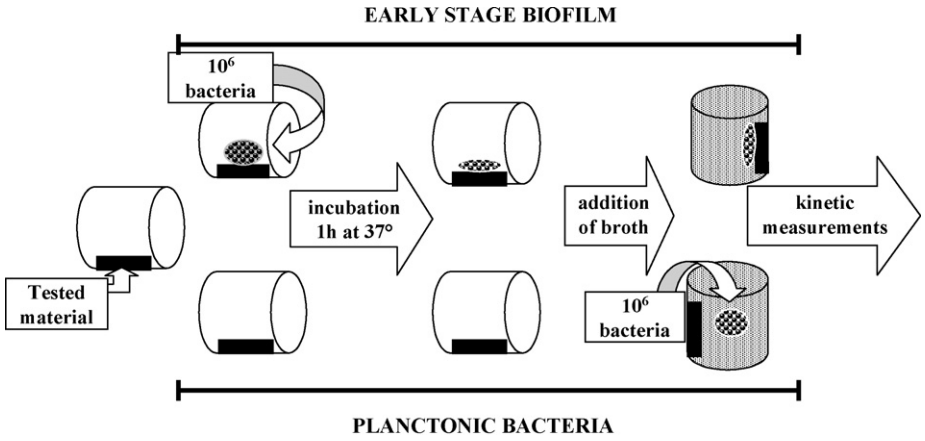

Fig. 1 – Schematic of direct contact test setup in a well of a 96-well microtiter plate. The sidewall of the well is coated with the tested material. To assess the effect on early-stage biofilm, bacteria are placed on the surface of the material. Incubation at 37 °C ensures bacterial interaction with the material. Another set of wells is used to test the effect on planktonic bacteria.

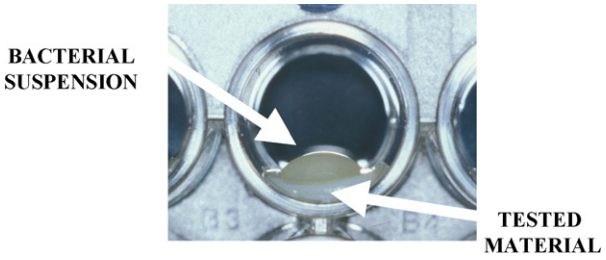

Fig. 2 – Photograph of bacterial suspension placed on tested material in well of 96-well microtiter plate, (as depicted diagrammatically in Fig. 1).

position for 1 h at 37 °C (Fig. 2). During that time, most of the suspension liquid evaporated ensuring direct contact between all bacteria and the tested surface, as shown by scanning electron microscopy (Philips 505 SEM at accelerating voltage, magnification 4000×; Fig. 3A). Then, 220 μl of growth medium were added to each well and the plate was incubated in a temperature-controlled microplate spectrophotometer (VERSAmax, Molecular Devices Corporation, Menlo Oaks Corporate Centre, Menlo Park, CA, USA), set at 37 °C, with 5 s mixing before each reading. The growth of bacteria shed from the biofilm was estimated by following the changes in OD_{650} every 20 min for 16 h. Scanning electron micrograph depicts the resulting 16 h biofilm (magnification 4000×; Fig. 3B). Experiments were repeated five times.

2.3.2. *Planktonic growth*

An additional set of seven wells coated with the test materials was used to evaluate the effect of the material on planktonic bacteria. For this purpose, each well was supplemented with 220 μl of BHI and inoculated with 10 μl of the above bacterial suspension. A 10 μl volume of the bacterial suspension placed on the uncoated walls of four wells in the same microtiter plate served as positive control. Four wells in the same microtiter plate, with tested material and with uninoculated BHI served as negative control.

2.3.3. *Aging of samples*

Test materials similarly prepared and placed in microtiter plates as above, were aged for 1 week at 37 °C. During this period, each well was filled with 250 μl PBS, which was replaced every 48 h. Before starting the DCT the PBS was aspirated and the plate was dried under sterile conditions.

2.3.4. *Calibration*

To allow inter-experimental comparison, each micotiter plate also included a set of wells for calibration of bacterial outgrowth. Ten microliters of the bacterial suspension was placed on the sidewalls of two uncoated wells, 275 μl of BHI broth was added and the plates were gently mixed for 30 s. A 55 μl sample from each well was transferred to an adjacent set of wells containing 220 μl of medium and the five-fold dilution was repeated six consecutive times.

2.3.5. *Bacterial growth in elute of the test materials*

The testing of soluble components, adds yet another facet to the antibacterial properties of the solid materials. The

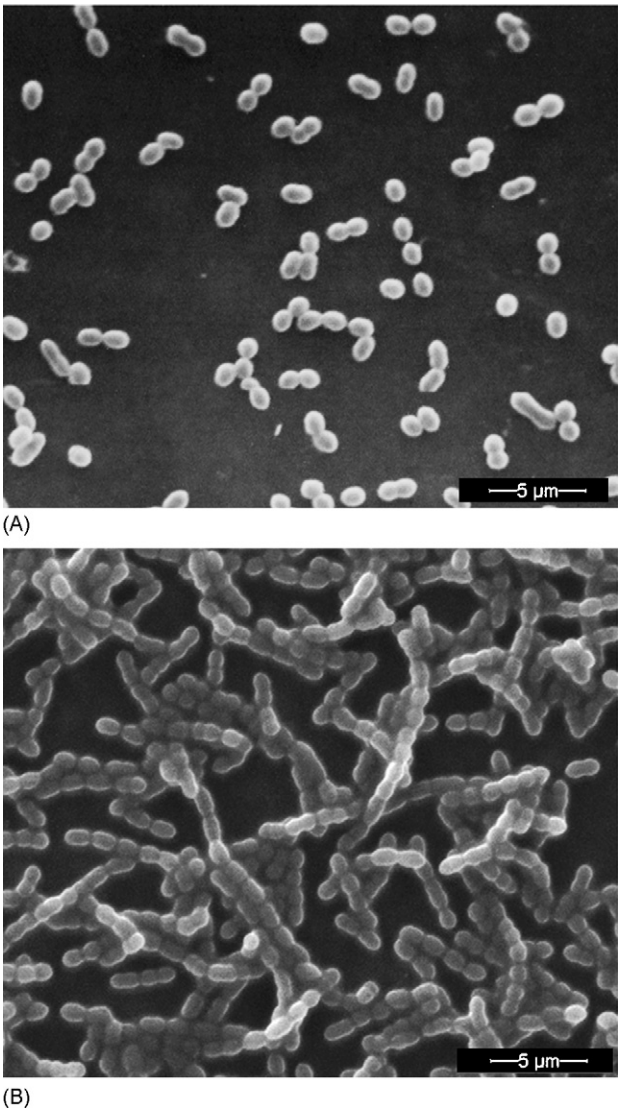

Fig. 3 – SEM micrograph (SEM 4000×) of *Streptococcus mutans* cells in direct contact with Z250, a dental composite resin. (A) Early-stage biofilm after 1 h incubation at 37 °C. (B) Bacterial growth and typical biofilm formed after 16 h incubation in temperature-controlled spectrophotometer.

antibacterial properties of the elute from the test materials on planktonic growth was tested quantitatively. Seven sidewalls were coated with each tested material, as described above, in a separate 96-well microtiter plate. Each well was supplemented with 230 μl of BHI and incubated for 24 h at 37 °C. A 220 μl volume from each well was transferred to an adjacent set of wells and 10 μl of a bacterial inoculum prepared as described above, were added, thus testing the effect of components eluted into the broth. The plate was placed in the temperature-controlled microplate spectrophotometer, set at 37 °C, with 5 s mixing before each reading. Bacterial growth was assessed by following the changes in OD_{650} every 20 min for 16 h.

The experiments described above were repeated three times.

2.4. Statistical analysis

The absorbance measurements were plotted, providing bacterial growth curves for each well in the microtiter plate. The linear portion of the logarithmic growth curve was used for statistical analysis. The results are expressed by two variables: the slope (a) and the constant (b) of the linear function $ax + b = y$, derived from the ascending part of the bacterial growth curve. The slope (a) and the constant (b) correlate with growth rate and the initial number of viable bacteria, respectively. The data were analyzed by one-way ANOVA, and the Tukey multiple comparison test. The level of significance was determined as $p < 0.05$.

2.5. Agar diffusion test (ADT)

S. mutans (200 μl; OD_{650} 0.6) was spread on mitis salivarius agar (MSB) (Difco, Detroit, MI) supplemented with bacitracin 0.0625 g/ml (Sigma) and *A. viscosus* (200 μl; OD_{650} 0.6) was spread on BHI agar. Disks of the test materials were prepared using a Teflon template (5 mm diameter 3 mm height). The composite resin materials were polymerized according to the manufacturers' instructions and amalgam samples were allowed to set for 30 min before testing. The four test materials disks were placed in 5 mm diameter punched holes in the agar. The plates were incubated for 48 h at 37 °C and the inhibition zone around each specimen was measured. Triplicate plates were prepared for each experiment.

3. Results

3.1. Kinetic measurements of bacterial growth

3.1.1. Direct contact test—early stage biofilm

S. mutans growth determined by absorbance (A_{650}) in a 96-well microtiter plate is shown in Fig. 4A. Each point on the curves is the mean value measured in seven wells containing the same tested material. The standard deviation of the measurements did not exceed 7% of the absolute values. The three composite resins (Z250, P60 and Tetric Ceram) showed a decrease in the logarithmic growth rate of *S. mutans* shed from the early-stage biofilm compared to the control. No growth of shed bacteria from amalgam was detected.

3.1.2. Aging of samples

Bacterial growth curves in similarly prepared microtiter plate after 1 week of sample aging showed an increase in *S. mutans* logarithmic growth rate on composite resins when compared to the control. No growth in the early-stage biofilm on aged amalgam samples was detected (Fig. 4B).

3.1.3. Early biofilm versus planktonic bacteria

Analysis of the data obtained for both the bacteria shed from the early biofilm and planktonic bacteria—is shown in Fig. 5A–D. The data are expressed as the slope and as the intercept of the regression line plotted from the logarithmic growth phase. The slope and intercept values were expressed as percent of controls, normalized to 100%:

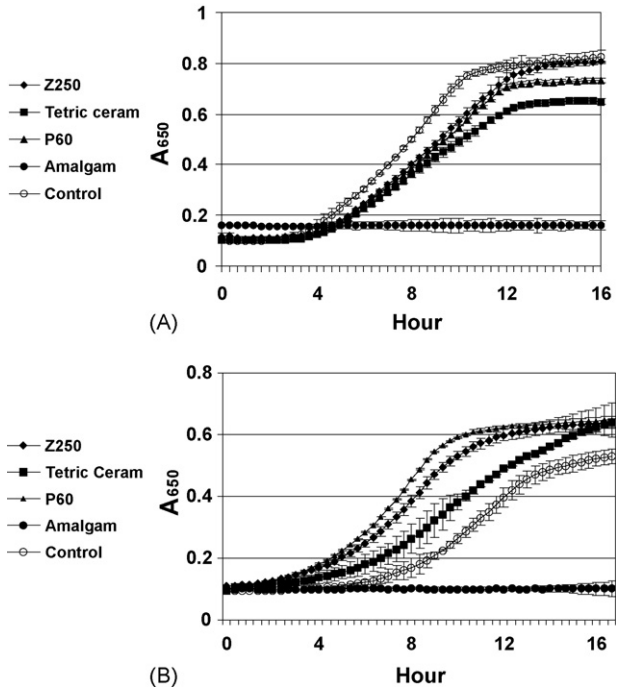

Fig. 4 – Kinetic measurements of bacterial growth following direct contact between *S. mutans* and four freshly prepared (A) and (B) 1 week aged dental restorative materials. The growth of the shed bacteria from the biofilm was measured every 20 min for 16 h. Each point on the curve is the mean absorbance (A_{650}) measured in seven wells similarly prepared in the same microtiter plate.

1. The slope and intercept values for amalgam, both in early-stage biofilm and in the planktonic, were nearly zero, that is, no *S. mutans* and no *A. viscosus* growth was measured in the amalgam-containing wells ($p < 0.0001$).
2. The values of the regression lines were lower in early-stage biofilm compared to planktonic bacterial growth ($p < 0.05$).
3. The regression line values obtained from the three composite resins varied significantly ($p < 0.05$); Tetric Ceram consistently yielding the lowest values (Fig. 5A–D).

3.1.4. Calibration

A gradual and reproducible decrease in optical density correlated with the serial dilution performed, indicated that the initial number of viable bacteria had no effect on growth rate or on the final optical density in the stationary phase (data not shown).

3.1.5. Bacterial growth in elute of the test materials

The antibacterial properties of the soluble components released from the test materials were expressed as percent of control (control = 100%) (Table 1). The bacterial growth of *S. mutans* in broth containing elutes from P60 or Z250 were similar to that of the control samples, while a slight decrease

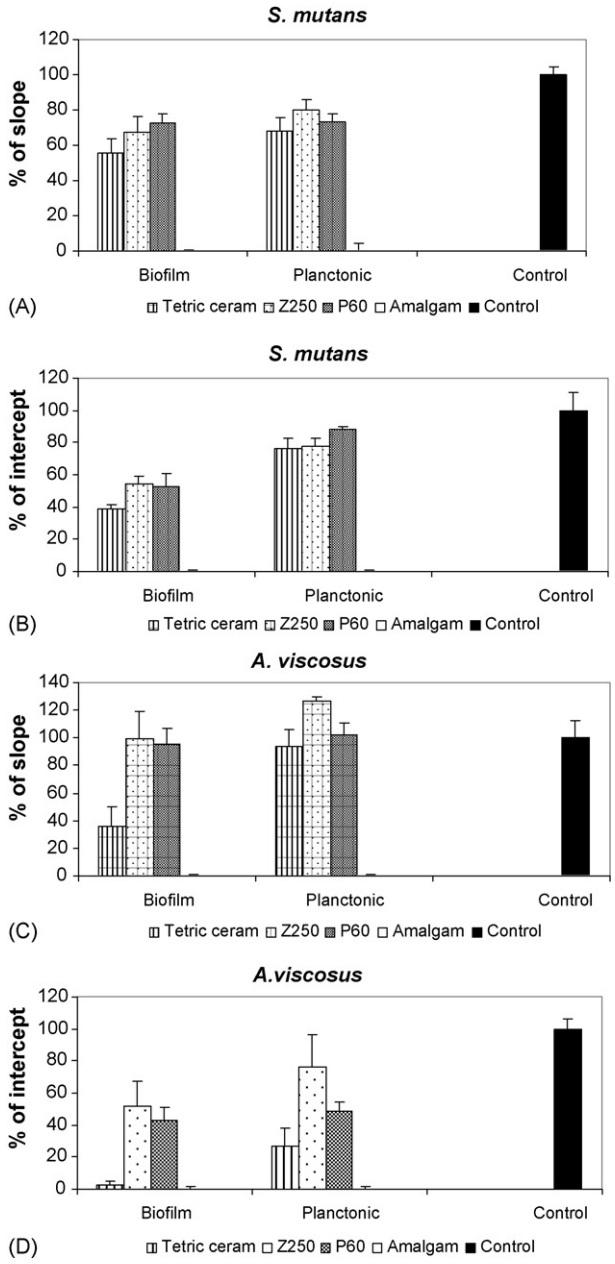

Fig. 5 – Analysis of bacterial growth kinetics. The linear portion of the logarithmic growth curve was used for statistical analysis. The results are expressed by two variables: the slope (*a*) and the constant (*b*) of the linear function $ax + b = y$, derived from the ascending part of the bacterial growth curve. The slope (*a*) and the constant (*b*) correlate with growth rate and the initial number of viable bacteria, respectively. The values are normalized to percent controls. The slope—correlate with growth rate; the intercept—correlates with initial number of bacteria. (A and B) *S. mutans* and (C and D) *Actinomyces viscosus*.

in growth was detected in the Tetric Ceram eluted broth. No growth was detected in the amalgam elute. The bacterial growth of *A. viscosus* was similar to that of the control in all test materials.

Table 1 – Effect of soluble components on *S. mutans* and *A. viscosus* growth

Bacteria	Tested material	ADT[a] (mm)	Bacterial growth in elute[b]
S. mutans	Amalgam	1.5 (0.5)	0
	Tetric Ceram	0	95 (3)
	Z250	0	98 (2)
	P60	0	99 (1)
A. viscosus	Amalgam	0	98 (2)
	Tetric Ceram	0	98 (2)
	Z250	0	98 (2)
	P60	0	98 (2)

[a] Inhibition halo diameter was measured in two perpendicular lines and the diameter of the tested sample was subtracted.
[b] Bacterial growth in the presence of soluble components eluted from the test materials for 24 h into the growth media were expressed as percent of control (control = 100%).

3.2. *Agar diffusion test*

No inhibition zone was detected around the samples of the three composite resin materials in the two test bacteria (Table 1). A halo of 1.5 ± 0.5 mm was measured around the amalgam samples in the *S. mutans* plates only.

4. Discussion

In the present study, we used several test conditions to quantitatively analyze the antibacterial properties of amalgam and composite resins. Kinetic measurements of bacterial growth were tested using the DCT.[15] In DCT bacteria are allowed to come in direct contact, under controlled conditions, with the tested material, to study the kinetics of bacterial growth.

We improved the DCT to include: (i) standardization of the tested material sample by weight and surface area, (ii) kinetic measurements of planktonic bacterial growth compared to shed bacteria from early-stage biofilm in the same experiment and (iii) verification of bacteria-material contact using SEM.

Amalgam completely inhibited the growth of both tested bacteria. This strong effect lasts for 1-week at least. In contrast, freshly polymerized composite resin materials exhibited only a reduction in replication rate of both bacteria. This effect was of short duration. During the 1-week aging, there was a turnabout and an accelerated growth rate compared to plastic surface (polycarbonate) control was observed. These findings support previous observations according to which, composite resin restorations tended to accumulate more bacteria and dental plaque *in vitro*,[16,17] and *in vivo*[7,18,19] than did other restorative materials.

The standardization of each tested group to a proper control showed that bacterial growth following direct contact are affected substantially more than planktonic bacteria. In all test materials the logarithmic growth rate was lower in early-stage biofilm compared to planktonic bacterial growth. Although many studies have dealt with the antibacterial effect of solid dental materials, few have focused on their differential effect on biofilm versus the planktonic phase. This is of particular importance in view of the fact that conversion

from the planktonic phase to the biofilm phase is a critical step in the development of infection.

Comparison of the results obtained from early biofilm with planktonic growth indicated a possible antibacterial effect of soluble components. Therefore, we performed two additional tests: (i) the ADT and (ii) the effect of the soluble components in the 24 h elute. Soluble components from amalgam and Tetric Ceram affect *S. mutans* growth, whereas *A. viscosus* growth is not affected by the soluble components in all test materials. The quantitative results of the effect of the elute on bacterial growth correlated with the qualitative ADT. Interestingly, we found that assays for soluble materials were negative, whereas the DCT or testing planktonic bacteria showed high antibacterial activity as in the case of amalgam and *A. viscosus*. Similar observation although less dramatic was found in the interaction of *A. viscosus* or *S. mutans* and freshly polymerized Tetric Ceram.

Collectively, our data indicate that antibacterial components may diffuse from solidified restorative materials and these components can be assessed either by ADT or measured by testing the elute. Other antibacterial components of poorly soluble restorative materials may be measured only by DCT. It follows that the assessment of solid materials has to employ more than one assay in order to characterize its antibacterial properties. Although in the present study we used pure cultures only, it is reasonable to assume that the differences found between the test materials may offer an explanation for the differences in plaque accumulation and the longevity of the restorations. *S. mutans* and *A. viscosus* present in human dental plaque are frequently associated with tooth decay.[20,21] These bacteria are also found attached to the surfaces of restorations and to the interface between the tooth and the restoration and may have crucial consequences in dentistry. Thus, aesthetic restorative materials still need to be improved regarding their antibacterial properties.

5. Conclusions

1. Amalgam showed a potent antibacterial effect in all tests used, and the effect lasted for more than 1 week.
2. The effect of the composite resins on bacterial growth was minimal and limited to a few days only.
3. One week aged composites promoted growth of *S. mutans* and *A. viscosus*.
4. The present findings may offer an explanation for the observed clinical increased biofilm accumulated on composites restorations.
5. It follows that the assessment of antibacterial properties of poorly soluble materials has to employ more than one assay.

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