Journal of Dentistry 123 (2022) 104209

Contents lists available at ScienceDirect

Journal of Dentistry

journal homepage: www.elsevier.com/locate/jdent

Antibacterial performance of composite containing quaternary ammonium silica (QASi) filler – A preliminary study

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ARTICLE INFO

Keywords:
Caries
Antibacterial composite
Quaternary ammonium
Composite
Antibacterial

ABSTRACT

Background: Antibacterial composite will have a significant clinical advantage in controlling caries. This study tests the antibacterial properties of a novel bulk-fill flowable composite (Infinx™, Nobio™ Ltd.) containing quaternary ammonium silica (QASi) filler particles.

Methods: Infinix™ was tested in-vitro by the direct contact test (DCT), using *E. faecalis* or whole saliva as inoculum. A similar formula composite without QASi served as a control. In addition, composite test samples were polymerized on three volunteers' intact buccal enamel surfaces of mandibular first premolars in a split-mouth design experiment. Traditional composite served as control (Filtek Bulk Fill Flowable, 3M). Bacterial viability on the composite surfaces weres assessed ex-vivo microscopically six months later, using a fluorescent dead/live stain. Images of each bacterial sample were taken using a fluorescent microscope (Nikon Eclipse 80i), and further live/total cell analysis was performed using ImageJ software.

Results: Following direct contact with one week of aged Infinix, more than 1 million *E. faecalis* bacteria were killed. Similarly, when using the saliva as inoculum, no single microorganism survived. Six-month in-vivo experiments supported these results by showing a reduction of 54%, 30% and 28% in live/total number of bacteria ratio retrieved from antibacterial composite vs. the control in volunteers #1, #2, #3 respectively.

Conclusion: Within the limitations of the experimental design, the present study suggest that antibacterial activity of quaternary ammonium silica particles (QASi) is comparable to that of previously described quaternary ammonium polyethyleneimine particles (QPEI). In addition, whole saliva bacteria are effectively killed by QASi-containing composite in-vitro and in-vivo, for a period of six month at least. Long-term full-scale clinical study is needed to confirm the findings of the present study and their implication on maintaining health balance. Clinical significance: Antibacterial composites containing QASi filler is a novel class of restoratives that may contributes to caries lesion control.

1. Introduction

Placing a restoration corrects the damaged tooth but has little or no effect on controlling caries disease or preventing recurrent cavities [1, 2].

Amalgam was the most commonly used material for restoring teeth affected with caries in the 20th century. However, amalgam has been in constant decline and shifted toward composite materials in recent decades. This shift is driven by increasing demand for aesthetics and the phase-down of mercury-containing compounds, including amalgam [3].

Composite materials have numerous shortcomings and limitations, rendering their use highly technique-sensitive [4]. It is observed that bacteria are unlikely to grow on amalgam, unlike composites that promote accretion and even accelerate bacterial growth. Analyzing the antibacterial activity of both composite and amalgam showed that amalgam has a potent antibacterial property that lasts for days, as opposed to composite, that is ineffective and even may support bacterial growth [5].

Cochrane review revealed that posterior composite restoration has a significantly higher risk for failure than amalgam restoration (RR 1.89, 95% P<0.001), and the leading cause was secondary caries [6]. In addition, a retrospective study in a public clinic reported nearly fourfold

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https://doi.org/10.1016/j.jdent.2022.104209
Received 22 April 2022; Received in revised form 18 June 2022; Accepted 23 June 2022
Available online 24 June 2022
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higher failure rate for posterior composite restoration compared to amalgam [7].

New antibacterial composites are under development in an attempt to cope with secondary caries-related failures. These composites are using one of the following strategies: [i] inclusion of antibacterial components such as quaternary ammonium (QA) into new monomer molecules [8–17], [ii] incorporation of small molecules into slowly releasing nano-fillers particles [18–21], and [iii] the use of non-releasing antibacterial filler nanoparticles of quaternary ammonium polyethyleneimine (QPEI) [22]. For the latter, we showed that even after six months of in-vitro aging, the anti- *S.mutans* properties were as effective as in the first day [22]. Furthermore, a recent in-situ study showed that composites with 1.5% (wt/wt) antibacterial quaternary ammonium silica (QASi) particles (Infinx™ Nobio™, Kadima, Israel) significantly reduced demineralization in enamel over four weeks in comparison to a conventional composite [23].

Previous studies showed broad spectrum inhibitory effect of QPEI particles on *S. mutans, E. faecalis, S. aureus, E. coli* and *L. casei*, as well as whole saliva bacteria [24,25,26] which represent the wide variety of species present in the oral cavity.

We hypothesize that antibacterial activity of QASi particles is comparable to the broad-spectrum QPEI particles activity.

The present study aimed to assess the *in-vitro* and *in-vivo* antibacterial activity of a new bulk-fill flowable composite containing 1.5% (wt/wt) quaternary ammonium silica (QASi) filler particles.

2. Methods

2.1. Materials

A new bulk-fill flowable composite (Infinix™ Nobio, Ltd, Kadima, Israel) containing 1.5% QASi filler was tested for its antibacterial properties. Unbound QA is cleared during the production of QASi to ensure no *in-situ* leakage of quaternary ammonium in the final product. As a control for the *in-vitro* experiments, a similar composite formulation without the QASi filler was produced (Nobio Ltd). As a control in the *in-vivo* investigation, we used Filtek™ Bulk Fill Flowable (3M ESPE, St. Paul, MN, USA).

2.2. Bacteria and inocula

We used *Enterococcous faecalis* ATCC 700802 in the *in-vitro* study as this relatively resistant bacterium is often found in the oral cavity, associated with pathologies [27, 28]. The direct contact test (DCT) was originally described with *E. faecalis* as it grows well and reproducibly in a temperature-controlled spectrophotometer, on which DCT is based on [28]. Bacteria were grown overnight aerobically in brain heart infusion broth (BHI) 37°C. Bacteria were harvested by centrifugation, resuspended in BHI, and adjusted to an optical density of 0.65 (at 650nm), equivalent to $\sim 3\times10^8$ cfu/ml [26].

In the second experiment, we used salivary bacteria as test inoculum. The whole saliva was collected and pooled from four healthy volunteers (age 25 to 58) and applied immediately on the surfaces of composite samples for testing by DCT (see below).

2.3. Direct contact test (DCT)

The in-vitro antibacterial effect was determined quantitatively with the DCT, as described by Weiss I E et al. and Beyth N et al. [28, 26]. Each composite sample was placed on the side-wall of 8 wells (n=8) in a 96-well flat-bottomed microtiter plate (Nunclon, Nunc; Thermo Fisher Scientific Inc.), and photo-polymerized. We tested the following surfaces: (i) composite incorporating 1.5% wt/wt QASi particles, (ii) control composite without QASi, and for comparison, (iii) microtiter plate plastic (polystyrene) surface. Samples were not polished as polymerized samples in microtiter wells cannot be polished. On each tested surface, we placed a ten μL bacterial suspension ($\sim 3\times10^6$ cfu). The plate was incubated vertically for one hour at 37°C to allow the suspension's liquid evaporation so each bacteria could contact the tested surfaces directly. After that, we added a growth medium to each well and incubated the plate in a temperature-controlled microplate spectrophotometer, set at 37°C, with 5-sec mixing before each reading. In the second experiment, as described above, ten μL of pooled whole saliva served as bacterial inoculum in a similar set-up 96-well microtiter plate.

Bacterial growth was monitored and recorded by continuous measurement of the changes in optical density (650nm) in each well every 20 min for 20h. The absorbance measurements were plotted, providing bacterial growth curves for each well on the plate. Each point in the growth curve (Fig. 2a) represents the average of 8 wells, independently measured at each time point.

For the calibration of each experiment in each microtiter plate, the same inocula of bacteria were inoculated in triplicate wells. Then a nine-fold dilution was performed seven times, in triplicate, resulting in numerous growth curves, until no bacteria were present, as indicated by a flat line (Fig. 2b). Each point in the growth curve represents the average of 3 wells, independently measured at each time point (Fig. 2b).

2.4. Aging of polymerized composite samples

Composite samples placed in microtiter plates, as above, were aged for one week to simulate performance in aqueous environment and to allow diffusion of unpolymerized composite residues. Each well was filled with 250μL phosphate-buffered saline (PBS) and replaced every 48 h. Before performing the DCT, PBS was discarded, and the plates were dried under sterile conditions. Each experiments were repeated at least twice.

2.5. In-vivo evaluation of antibacterial composite

Composites were tested in a split-mouth design in 3 healthy volunteers. The study was approved by the Institutional Review Board (# 60.19). Composite samples of +/- 2×2×1mm were light polymerized on the intact buccal enamel surfaces of mandibular first premolars of the volunteers after 15 seconds of etching and priming and bonding according to manufacture instructions. The antibacterial composite was placed on one side and the control composite on the contralateral side. Following polymerization, samples were finished using polishing discs (medium and fine, 3M™ Sof-Lex Contouring and Polishing Discs). Volunteers were instructed to continue their routine oral hygiene protocol. Six months later, biofilm was collected from the surfaces of each composite sample with a dental micro-brush (Fig. 1, a+b). Before biofilm collection, the teeth with the composite samples were rinsed with tap water using a dental triple syringe for 5 seconds to remove loosely-bound bacteria and food remnants.

Biofilm bacteria were spread immediately on glass slides and stained with 5μl of a BacLight live/dead assay kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The slides were kept in the dark at room temperature for 20min. Images of each sample were captured with a fluorescence microscope (Nikon Eclipse 80i, Nikon Corp. Tokyo, Japan), and live/dead cell image analysis was performed using Image J 1.44 software (image J nih.gov) USA. The average live/total number of bacteria ratio measured from 10 images was calculated on each side for each individual. A paired two-sample t-test was used to analyze the results (significance level: $p < 0.05$).

3. Results

3.1. In-vitro antibacterial DCT

The results of microtiter plate aged for 7 days are shown in Figs. 2a and b. The value of each point on the bacterial growth curves is the mean absorbance (A_{650}) measured in 8 wells similarly prepared in the same

Fig. 1. Composite samples on mandibular first premolars: (**a**) Control composite on the right lower first premolar, (**b**) Antibacterial tested composite on left lower first premolar. After six months, the biofilm was collected with a dental micro-brush and spread on a microscope glass slide for BacLight live/dead assay.

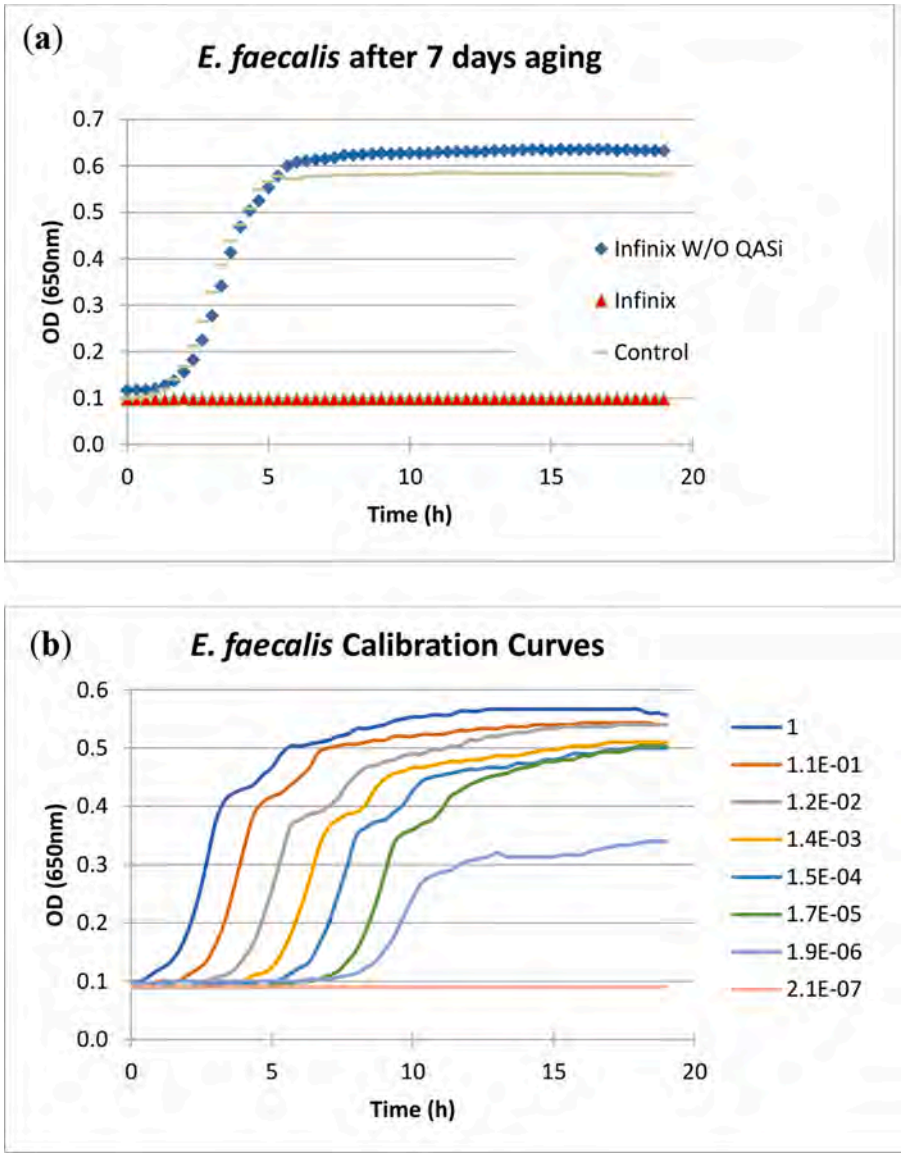

Fig. 2. (**a**). Bacterial growth kinetics following direct contact with *E. faecalis* after seven days of aging. Each point on the curve is the mean absorbance (A_{650}) measured in 8 wells, similarly prepared in the same microtiter plate. Composite without (W/O) QASi nanoparticles - blue; control polystyrene - green; and Infinix composite with QASi nanoparticles - red.
(**b**). *E.faecalis* calibration curves. Ninefold dilutions were performed seven times in triplicate, resulting in 8 growth curves in the same microtiter plate. Each point on the curve is the mean absorbance (A_{650}) measured in 3 wells.

microtiter plate. The standard deviation of the values measured in the DCT of the 8 wells, were ranging between 0.02 and 0.08 optical density (OD), similar to the distribution described in previous publications [24, 28]. The ninefold dilutions (Fig. 2b) yielded respective calibration growth curves, from which the actual number of viable bacteria at the beginning of the experiment is calculated.

The DCT shows that the composite containing QASi particles inhibite *E.faecalis* growth; a straight line in the growth curve after 24 hours of incubation indicate no bacterial growth (Fig. 2a). Based on the calibration curves (Fig. 2b), at least 1 milion live cells were inoculated. Thus even if one single bacteria would survive, it will show an onset of growth, 8-10 hours after the beginning of the experiment. In contrast, at least 10^6 viable bacteria grew on the surface of the same formulation control composite lacking the QASi particles and on the plastic (polystyrene) surface (Fig. 2a).

In the second in-vitro experiment, we used the whole saliva pooled

from 4 volunteers as the bacterial source of inoculum for DCT. Saliva bacterial growth kinetics, following direct contact with QASi–containing composite, and with control composite (without QASi) are shown if Fig. 3. Each point on the curve is the mean absorbance (A_{650}) measured in 8 wells every 20 min. Direct contact of whole saliva bacteria with control composite result in typical bacterial growth curve, while with antibacterial composite complete growth inhibition of all bacteria is measured (Fig. 3).

3.2. *In-vivo: live/dead assay*

The in-vivo effect of the antibacterial composite was tested in 3 volunteers by evaluating the live/ total bacterial ratio in each image captured under a fluorescence microscope. Representative live/dead stained biofilm samples collected from the composite surfaces are shown in Fig. 4. Biofilm composed of mainly dead cells is stained in red while biofilm composed of mainly live cells is stained in green (Fig. 4 a and 4 b respectively).

The live/total number of bacteria ratios on the antibacterial composite and the control composite after six months in the oral cavity were retrieved from up to 10 images. The average live/total number of bacteria ratio for each patient was calculated (Fig. 5). Volunteers #1, #2, and #3 showed low live to the total number of bacteria ratios on the antibacterial composite (Infinix; blue) compared to the control composites (Filtek; red).

In bacterial samples collected from QASi-containing composite in volunteer #1 there was a reduction of 54% in live/total number of bacteria (paired two-samples t-test, $p<0.0001$). In bacterial samples collected from QASi-containing composite in volunteer #2 and #3 there was a reduction of 30% ($p<0.01$) and 28% ($p<0.001$) in live/total number of bacteria respectively.

4. Discussion

Previously, Byth et al. showed that polyethyleneimine-based particles with bound non-releasing antibacterial moieties could result in a broad spectrum and long-lasting antibacterial composites [22]. The present study tested QASi particles in which silica filler replaced the polyethyleneimine core. Here we show that composite resin containing QASi filler particles has similarly highly potent broad-spectrum antibacterial properties. Using *E. faecalis* as a test bacterium, the reduction was one million viable bacteria at least. This number was the maximal bacterial inoculum that could be tested; as no bacteria survived on the restorative material surface, we assume that the surface-antibacterial activity is even more substantial.

Furthermore, this result is achieved after seven days of aging in an aqueous environment, indicating the stable nature of the antibacterial QASi filler in the composite. Aging that simulate chemical and mechanical wear of samples was beyond the scope of this study. Here, the purpose of the aging was to allow unpolymerized composite residues to diffuse into aqueous solution. It is noteworthy that in many clinical situation, such as class V restorations, clinicians do not polish the restoration, and unpolymerized residues (oxygen-inhibited layer) exposed to the oral environment is common practice.

For decades, studies tested a range of composite restoratives used in dentistry for their antibacterial activity using agar diffusion and direct contact tests [29–33]. Not surprisingly, none of the composites possessed antibacterial properties. Moreover, bacteria grew on some composites samples even better and faster than on the inert plastic (polystyrene) control surface.

Hundreds of bacterial species are present in whole human saliva [34, 35], the composition of which varies from one individual to the other. We used pooled saliva collected from 4 volunteers as the source for the bacterial inoculation in the DCT, and showed that all the microorganisms were killed. This result is another example of the remarkable broad-spectrum antibacterial activity of the QASi-filler containing composite. Saliva bacteria contacting composites in-vitro simulates only the early in vivo events immediately after bacterial attachment. In the controlled conditions of DCT we can assure direct contact of all bacteria with the tested composite surfaces, therefore, complete elimination of living bacteria was achieved under these in-vitro conditions.

For the in-vivo experiment, we choose to bond the samples to lower premolar teeth, as they are more protected from the friction of the buccal mucosa by the upper premolars, because of the over jet. We wanted to evaluate the in-situ antibacterial activity of QASi containing composite close to regular conditions. Therefore, we instructed volunteers to brush their teeth and consume their diet normally.

There are some limitations to the experimental set-up. Under these conditions we could not assure direct contact of all saliva bacteria with the composites, and therefore, bacteria not contacting the surface, survived. Furthermore, saliva bacteria may proliferate on antibacterial composite, if they do not contact directly the composite. Another limitation of this experiment is the sampling of the surface by micro brushes, by which removal of all bacteria cannot be assured, thus many bacteria may remain on the composite, and this may bias the results.

Nevertheless, the in-vivo experiments exemplify the potentially

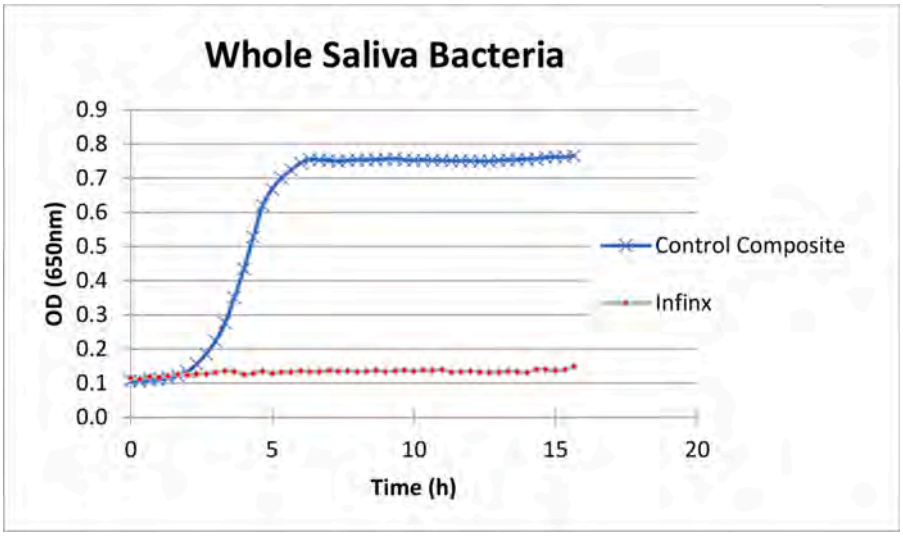

Fig. 3. Bacterial growth kinetics, following direct contact with saliva bacteria. Each point on the curve is the mean absorbance (A_{650}) measured in 8 wells every 20 min. The absorbance of the antibacterial composite with QASi is shown in red squares, and the control composite (without QASi) is in blue.

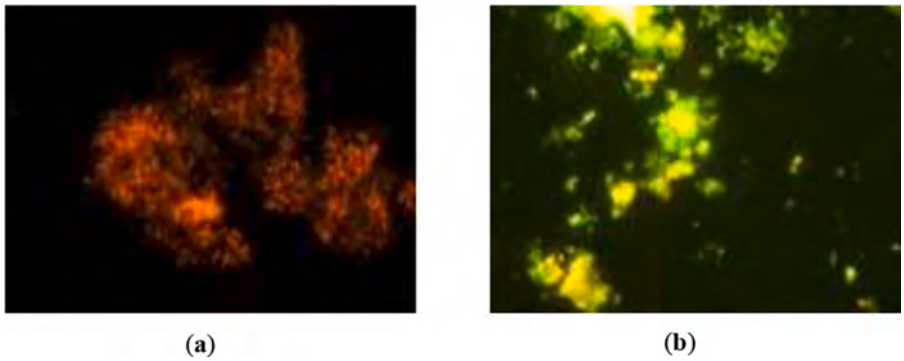

Fig. 4. Examples of fluorescence microscope images of live/dead stains of biofilm bacteria collected from composite samples (shown in Fig. 1) after six months in the oral cavity. (a) biofilm showing mainly dead cells stained in red. (b) biofilm showing mainly live cells stained in green.

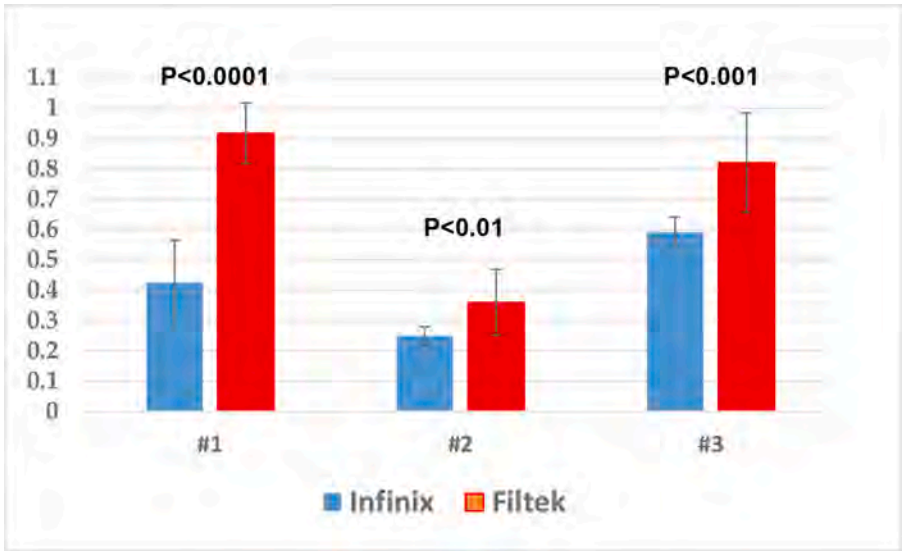

Fig. 5. Average live/total ratios of the number of bacteria for each volunteer (#1, #2, and #3) on the antibacterial composite (Infinix; blue) and the control composite (Filtek; in red) after six months in the oral cavity. The live/total ratios were retrieved and calculated from images captured with a fluorescence microscope (paired two-sample t-test, significance level: $p < 0.05$).

broad-spectrum and lasting antibacterial effect of the QASi filler even in a harsh oral environment.

Caries disease is best controlled by changing the caries balance. Change in balance is partly achieved by using protective factors such as a 0.12% chlorhexidine rinse or a 5,000 ppm fluoride-containing toothpaste [1,2,36]. In an in-situ clinical study, Rechmann et al. [23] showed that composites with QASi antibacterial particles significantly reduced demineralization in enamel adjacent to a 38-μm gap over four weeks in comparison to a conventional composite. Their study demonstrates the in vivo effect of antibacterial activity on the very beginning of the caries process, hypothesizing that composites with QASi filler particles have the potential to reduce the occurrence of secondary caries. With these results collectively, it is reasonable to assume that restorative materials, which possess stable broad-spectrum and long-lasting antibacterial properties, may play a significant protective role in shifting the caries balance towards health.

Except for Infinix, all restorative materials in clinical use for filling cavities have little or no antibacterial properties. Among those who exhibit a certain degree of antibacterial activity, the activity fades rapidly within days [29–33]. Therefore, we suggested that the antibacterial surface property of the QASi-containing composite bears the potential to play an essential role in controlling primary and secondary caries by killing causative microorganisms at the most susceptible site and thus reducing demineralization. Long-term full-scale clinical study is needed to confirm the findings of the present study and their implication on maintaining health balance.

It was reported that the addition of antibacterial particles to materials, such as resin-based restorative materials [37], poly-methyl methacrylate-based orthopedic cement [38], epoxy-based endodontic cement [39], temporary cement, zinc oxide eugenol, or calcium sulfate [40] resulted in antibacterial surface properties. Based on the present and other studies [23, 38-41], we postulate that QASi particles will contribute significantly to the antibacterial properties of a variety of other dental and medical devices.

Funding

This research received no external funding. Composite materials were supplied by Nobio Ltd.

CRediT authorship contribution statement

Michal Dekel-Steinkeller: Conceptualization, Methodology, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Ervin I. Weiss:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing. **Trudi Lev-Dor Samovici:** Methodology, Investigation, Data curation. **Itzhak Abramovitz:** Methodology, Writing – original draft.

Declaration of Competing Interest

M Dekel – Steinkeller is a consultant for 3M and for Nobio
E.I. Weiss is the founder, stock holder and board member of Nobio
All other authors declare no conflict of interest

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