

Leakage of food-borne *Enterococcus faecalis* through temporary fillings in a simulated oral environment

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Abstract

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Aim To evaluate the hypothesis that food-borne viable *Enterococcus faecalis* cells could enter the root canal space via coronal leakage.

Methodology In a simulated oral environment under mastication the capacity of a calcium sulphate-based temporary filling material (Cavit W) to prevent leakage of *E. faecalis* from a cheese through the endodontic access cavity into the pulp chamber was assessed. Standardized class I access cavities were prepared in human maxillary molars. These were filled with Cavit of either 2 or 4 mm thickness ($n = 16$, each). Empty access cavities served as positive, teeth filled with a light-curing composite material acted as negative controls ($n = 8$, each). A cheese containing viable *E. faecalis* cells was placed on the occlusal aspects of test and control teeth, which were subsequently

subjected to 680 mastication loads per day for 1 week in a masticator device perfused with artificial saliva at 37 °C. Leakage of *E. faecalis* from the cheese into the pulp chamber was assessed by culture on a kanamycin aesculin azide agar and compared between groups using Fisher's exact test.

Results All of the positive controls showed pure growth of *E. faecalis*. In addition, one of the negative control teeth leaked. The 4 mm application of Cavit prevented leakage of *E. faecalis* significantly better than the corresponding 2 mm application: 1 of 16 specimens compared with 6 of 16 specimens had leakage, respectively ($P < 0.05$).

Conclusions The current results substantiate the suspicion that food-derived microbiota could enter the necrotic root canal system via microleakage.

Keywords: *Enterococcus faecalis*, cheese, temporary filling, microleakage.

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Introduction

The endodontic literature is replete with reports on the occurrence of enterococci in filled root canals (Portenier *et al.* 2003). It has always been held that microorganisms found in the root canal space are derived from the

taxa colonizing the oral cavity (Sundqvist 1994). However, that is not necessarily the case. The necrotic root canal system represents a unique ecological habitat in the mouth, with specific selection criteria for microbial growth (Sundqvist 1994). In teeth with vital pulps the endodontic system is sterile, and becomes infected gradually as the pulp loses its viability and consequently, its cell-mediated defence mechanisms (Langeland 1987). To colonize the necrotic root canal, it may not be a pre-requisite for a microorganism to be consistently present at other oral sites. Transient microorganisms that do not survive in the oral

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environment under healthy conditions may also enter the unsealed root canal space and potentially take part in microbial root canal aggregates.

One taxon that is commonly found in necrotic root canal systems, and even more frequently in filled root canals, is *Enterococcus faecalis*. It may be argued, however, that *E. faecalis* is not a permanent oral coloniser. Early reports that linked enterococci to different oral diseases such as caries (Orland *et al.* 1955) were hampered by the fact that before the 1970s, proper biochemical identification of enterococci was not possible, making it likely that *mutans* and other streptococci were mistaken for enterococci (Gold *et al.* 1975). *Enterococcus faecalis* strains recovered from the human mouth survived as monocultures in gnotobiotic rats, however, they could not maintain in the oral cavities of conventional rats (Gold *et al.* 1975). From the healthy human oral cavity, enterococci are rarely recovered (Sedgley *et al.* 2004, Aas *et al.* 2005). On the other hand, enterococci are commonly found in or on different food types for raw consumption, such as cheese, meat products, olives and vegetables (Franz *et al.* 2003). In a recent study, viable enterococci were found in all four of the investigated cheese types (Swiss Tilsiter, French soft cheese, Feta and Mozzarella). In a cohort of healthy dental students ($n = 50$), no viable enterococci were recovered from the oral cavities. After ingestion of Brie de Meaux containing a substantial number of viable *E. faecalis* cells, these were recovered in decreasing numbers over time for 100 min from the oral cavities of eight volunteers who refrained from eating or drinking. After 1 week of no intervention, normal diet and normal oral hygiene, none of the volunteers harboured enterococci in their oral cavities (Razavi *et al.* 2007). The transient nature of food-borne *E. faecalis* colonization of the mouth could explain the finding that *E. faecalis* is inconsistently recovered from the oral cavities within subjects between different experimental days (Williams *et al.* 1950). However, once they reach the necrotic root canal system, enterococci appear to survive both as monoinfectants and in combination with other microbiota (Fabricius *et al.* 1982), and withstand the environmental changes induced by a root filling (Fabricius *et al.* 2006). Interestingly, enteric bacteria including *E. faecalis* are more frequently found in root canal systems that were left unsealed between visits than in sealed counterparts (Sirén *et al.* 1997). Histologic studies have revealed food remnants in both root canal treated teeth and periapical lesions (Nair 2006). In addition to *E. faecalis*, other typically food-borne *Enterococcus* species such as

E. faecium and *E. casseliflavus* have been identified in necrotic human root canal systems (Ferrari *et al.* 2005). To what extent commonly used temporary filling materials could prevent the leakage of food bacteria such as enterococci into the root canal system during mastication, however, has not yet been investigated.

The goal of the current study was to evaluate the capacity of a commonly used calcium sulphate-based temporary filling material (Cavit W, Espe, Seefeld, Germany) to prevent coronal leakage of food-borne *E. faecalis* into the pulp chamber of human maxillary molars in a simulated oral environment under mastication *ex vivo*. Aliquots of a soft cheese with a relatively high *E. faecalis* content were applied on standardized class I cavities filled with test and control materials. Teeth were then subjected to masticatory loading for 7 days at 37 °C in simulated body fluid, and leakage of *E. faecalis* into the pulp chamber was monitored.

Materials and methods

Cheese

Two different brands of 'Brie de Meaux' were bought from local supermarkets in Zürich, Switzerland, and investigated for their enterococci content. To exclude contamination from the surface, 1-g pieces were cut out from the body of each cheese using sterile instruments. This and all the subsequent microbiologic laboratory procedures were exercised under aseptic conditions in a safety cabinet (SFE.120 EN; Skan AG, Basel, Switzerland) using sterile materials. Cheese pieces were homogenized for 1 min in 10 mL of a 2% sodium citrate buffer using a tissue homogenizer (Ultra-Turrax T8; IKA-Werke, GmbH & Co., Staufen, Germany). Subsequently, tenfold serial dilutions were prepared in 2% sodium citrate down to 10^{-5} . As much as 50 μ L of each dilution was plated on kanamycin aesculin azide agar (KAAA; Merck, Darmstadt, Germany) using a spiral diluter (Spiral Systems, Inc., Cincinnati, OH, USA) and incubated at 42 °C in a 5% carbon dioxide atmosphere (Domig *et al.* 2003). Colonies were counted after 3 days of incubation (Qamer *et al.* 2003). *Enterococcus* colonies were presumptively identified by their blackening of the agar due to hydrolysis of esculin. Representative colonies of all morphotypes were picked up and subcultured on Columbia blood agar (Oxoid, Basingstoke, UK) incubated as described above. Using a phase contrast microscope (Leitz Dialux 22, Leica, Basel, Switzerland) at

1000× magnification, Gram-positive coccoid bacteria growing in chains were identified. Subsequently, a catalase test was performed and Gram-positive, catalase-negative cocci growing in strings were adjusted to an OD₅₅₀ of 1, as assessed in a cuvette of 1 mm light path in a spectrophotometer (U 2001; Hitachi, VWR International AG, Dietikon, Switzerland), and identified to species level using a biochemical test kit (rapid ID 32 STREP; bioMérieux, La Balme les Grottes, France) according to the instructions of the manufacturer.

Determination of group size

Based on *in vivo* studies (Lamers *et al.* 1980, Beach *et al.* 1996), expected frequencies for leakage with 2 mm and 4 mm Cavit W temporary fillings was 0–10% and 30–40%, respectively. With expected frequencies of 5% vs. 35%, a group size of 16 per Cavit application depth appeared adequate to detect significant differences.

Tooth mount

Forty-eight intact maxillary molars stored in 0.1% thymol at 5 °C were selected from the department's collection of extracted teeth. The absence of caries and cracks was verified under a stereo dissecting microscope (Leica Wild M3Z; Wild, Heerbrugg, Switzerland) with an internal light source (intralux 4000; Sowo-Dent, Birmsdorf, Switzerland). Roots were resected 3 mm from the cemento-enamel junction. Subsequently, standardized occlusal cavities were drilled into the centre of the occlusal aspect of these teeth using an inlay preparation diamond bur (Cerana, JS Dental MFG INC, Ridgefield, CT, USA) under water-cooling. Standardized class I cavities of 4.5 mm depth, 4.8 mm occlusal diameter and 3.0 mm diameter towards the pulp chamber were obtained by introducing this standard bur level with the occlusal plain. Using a cylindrical diamond bur, the 3 mm opening towards the pulp chamber was extended to the resected root opening. Teeth were then each placed centrally into custom made stainless steel specimen carriers of 14 mm inner diameter and 3.5 mm depth. To fix the teeth, the inner space of the carriers containing the tooth was filled with a cold curing methyl methacrylate-based resin (Technovit 3040; Heraeus Kulzer, Wehrheim, Germany). The resin was polymerized in cold water to reduce reaction heat. When the resin had set, the tooth-resin unit was carefully extracted from the carrier.

Subsequently, teeth were divided into two similar test groups: 2 mm Cavit W, 4 mm Cavit W, plus negative

Table 1 Test and control teeth of the current study

Group	<i>n</i>	Seal in access cavity
Test 1	16	Cavit W, 2 mm
Test 2	16	Cavit W, 4 mm
Positive control	8	No seal
Negative control	8	Tetric Ceram

and positive controls using a computer algorithm (<http://www.random.org/>, Table 1). To ensure filling thickness, a putty mass was placed into the teeth (Crayola Mini Kids Dough, Binney & Smith, Bedford, UK) to the respective level measured from the occlusal edge of the preparation. Positive control teeth were left with an empty access cavity, whilst access cavities of the negative control teeth were filled with 4 mm of a light curing composite resin (Tetric, Ivoclar Vivadent, Schaan, Liechtenstein) applying an incremental technique according to the manufacturers guidelines. Composite fillings were bonded to enamel and dentine using a four-step bonding system (Syntac Classic, Ivoclar-Vivadent).

After placement of test and control fillings, the putty mass was carefully removed from the apical opening of the specimens, and teeth, specimen carriers, plus the self-adhesive aluminium foil (Migros, Zürich, Switzerland) and the cotton pellets to be used later on were sterilised in ethylene oxide. Subsequently, the sterilized materials were aerated for 2 days in a sterile humid environment. A sterile cotton pellet soaked in fluid universal medium (FUM, Gmür & Guggenheim 1983) was then placed inside the tooth specimens from the apical access, which was subsequently sealed with the self-adhesive aluminium foil. Finally, tooth specimens and carriers were connected and sealed using an insulating silicone glue (Acetoxysilane; Dow Corning 734, Dow Corning GmbH, Wiesbaden, Germany), which covered the whole lateral aspects of the teeth (Fig. 1). The seal was allowed to set for 2 h. All the procedures described in this section were carried out by one researcher.

Occlusal loading in simulated oral environment

A computer-controlled masticator device (CoCoM 2, PPK Zürich, Switzerland), consisting of six individual waterproof masticator units connected to a central 10-L liquid reservoir, was used (Göhring *et al.* 2003). The setup was disinfected by perfusion with 0.5% NaOCl solution for 2 h, followed by hypochlorite inactivation using sodium thiosulphate (4 g L⁻¹) and a flush with

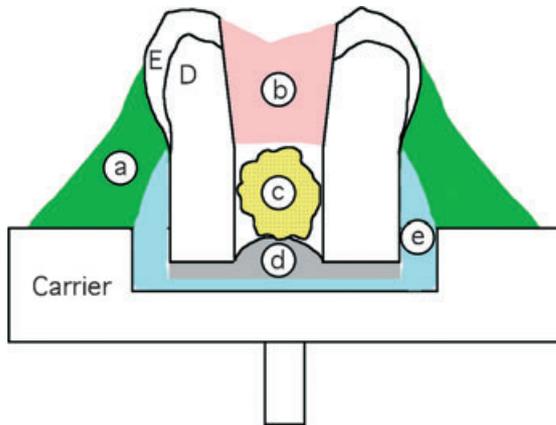


Figure 1 Schematic cross-section through the middle of a mounted specimen. E, enamel; D, dentine; a, silicone glue; b, test or control filling material; c, cotton pellet soaked in fluid universal medium; d, self-adhesive aluminium foil; e, methacrylate resin.

sterile water. Inactivation of the hypochlorite was checked by a standard iodine-thiosulphate titration method. Specimens were mounted against brass ball antagonists of 7.9 mm diameter (Fig. 2). The stainless steel carriers ensured a defined repositioning of specimens. They were mounted so that antagonists did not touch the fillings but rather the central cusp aspects under load. Cheese aliquots of 1.5 g were placed directly on the occlusal tooth surfaces. Subsequently, the system was flooded with sterile artificial saliva, which was maintained at 37 °C by an internal heating device. Artificial saliva was prepared according to the recipe for simulated body fluid first described by Cho *et al.* (1995). Sodium thiosulphate, water and artificial saliva were sterile-filtrated through a 0.2- μ m cellulose acetate filter (Sartorius AG, Göttingen, Germany). The artificial saliva was circulated and teeth were subjected to the physiologic equivalent of 680 mastication loads per day (DeLong *et al.* 1985) at a standard load of 49 N and a frequency of 1 Hz. Specimens were maintained in the masticator for 7 days. Thereafter, specimens were transported to the microbiologic safety cabinet and carefully removed from their stainless steel carriers. Aluminium foils covering the access to the FUM-soaked cotton pellet were disinfected with 2% NaOCl followed by thiosulphate inactivation. The foils were then perforated using sterile tweezers and cotton pellets were vortexed in microcentrifuge tubes containing 0.4 mL of sterile artificial saliva. Dilution series in artificial saliva were plated on KAAA and incubated for 3 days as described above.

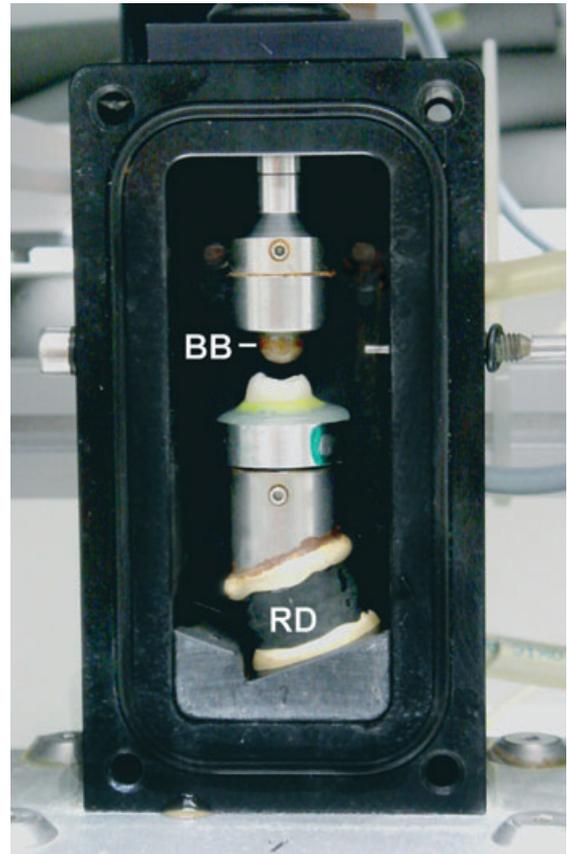


Figure 2 Masticator unit with mounted tooth specimen and brass ball antagonist (BB). Note the rubber damper (RD), which created a sliding motion during occlusal loading.

Sterility checks were performed in the preliminary test phase of this study, when each step was determined to consistently prevent contamination. The foil was used as an additional security measure; the silicone insulation of the teeth was already bacteria tight by itself and swab samples from the foil before perforation and even before hypochlorite disinfection were consistently negative.

Data analysis

Leakage between 2 and 4 mm Cavit W temporary fillings was compared using Fisher's exact test with the alpha-type error set at 0.05.

Results

One of the cheeses showed mixed cultures of *E. faecalis* and an unidentified species on KAAA. The other cheese

showed pure cultures of *E. faecalis* on KAAA, and was selected for the current study. It contained 80 000 viable *E. faecalis* cells per gram. The cheese was stored at 20 °C below zero, and aliquots were thawed for the individual experiments. Cultures of the frozen/thawed cheese showed that it maintained its content of viable *E. faecalis* cells.

Viable *E. faecalis* cells were recovered from the artificial saliva that circulated in the computer-controlled masticator device during the whole course of the experiment: a median value 3500, 1200, and 800 CFU mL⁻¹ was recovered from the liquid reservoir 1, 4, and 7 days after initiation of the experiment, respectively. The experiment was performed eight times with two teeth filled with 2 mm Cavit W, two filled with 4 mm Cavit W, one positive and one negative control. The wash protocol between individual experiments described here left no bacteria cultivable on KAAA nor any detectable traces of hypochlorite in the liquid circulation system.

Cotton pellets in teeth with open access cavities (positive controls) all showed pure growth of *E. faecalis* on KAAA. Amongst the negative control teeth sealed with the light-curing composite material, one showed *E. faecalis* leakage. The 4-mm application of Cavit W prevented leakage significantly better than the corresponding 2-mm application: one of 16 specimens compared with six of 16 specimens showed leakage, respectively ($P < 0.05$).

Discussion

Under the conditions of the current study, leakage of viable food-borne *E. faecalis* cells occurred in teeth with unsealed access cavities, as well as in some of the teeth sealed with temporary or even permanent filling materials. A 4-mm application of Cavit W provided a significantly better seal than a corresponding 2-mm application.

This would be the first study suggesting a link between the occurrence of enterococci in foods and their presence in the pulpless root canal space. The original Enterococcus habitat is the mammalian, especially human, gastrointestinal tract. However, enterococci colonize diverse habitats because of their exceptional capacity to grow in different environments such as soil, surface waters and fermented food (Giraffa 2002). Implications of the fact that many if not all food products sold for raw consumption contain viable bacteria, which could potentially affect the oral flora or become established in specific oral habitats, is a field

that has obtained but little attention thus far (Meurman 2005). A cheese type that is known for its almost consistent high content of *E. faecalis* (Franz et al. 2003) was chosen. On the basis of *in vivo* observations, the healthy oral cavity appears not to be an ideal long-term habitat for *E. faecalis* (Sedgley et al. 2004, Razavi et al. 2007). However, the rate of clearance of food-derived enterococci from the oral cavity is such that infection of predilection sites cannot be excluded (Razavi et al. 2007). If food-borne enterococci reach the unsealed necrotic root canal system, they may colonize that habitat. Clinical studies employing serological methods or DNA matching are indicated to further investigate this theory.

It must be cautioned that the current results were obtained in a simulated oral environment *ex vivo*, and no direct clinical conclusions may thus be drawn. Nevertheless, the results of the present study correlate well with findings on bacterial leakage through Cavit temporary fillings obtained under *in vivo* conditions. A 2-mm Cavit W temporary filling allowed microbial leakage in 37% of the investigated monkey teeth after 1 week (Lamers et al. 1980), which correlates with the 37.5% leakage observed in the current study. A clinical study in humans showed no microbial leakage through a 4-mm Cavit W temporary access cavity seal after 3 weeks (Beach et al. 1996), which is comparable with the 6% leakage rate reported here. Cavit is the temporary restoration most commonly used amongst the American endodontists (Vail & Steffel 2006). On the basis of the current results and clinical observations (Beach et al. 1996), this material can indeed provide an adequate access cavity seal if placed at sufficient

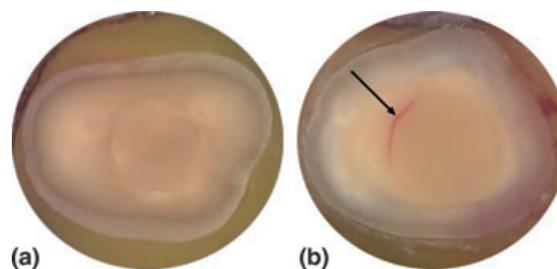


Figure 3 Two negative control specimens filled with a composite resin. The fillings were bonded to enamel and dentine, applied using an incremental technique, and light-cured according to the manufacturer's guidelines. Nevertheless, one of the eight negative control fillings allowed *E. faecalis* leakage into the pulp chamber. Whilst the other composite fillings allowed no leakage of a basic fuchsin dye (a), a marginal gap was visible in the leaking specimen (b, arrow).

thickness. However, it should be realized that Cavit merely postpones bacterial leakage, and cannot prevent it in the long term (Barthel et al. 2006). Furthermore, as Cavit remains relatively soft after setting, it should only be used to seal access cavities of limited size, without contact with the antagonizing tooth (Beach et al. 1996).

The somewhat unsuspected finding that one of the negative control specimens in the current study allowed *E. faecalis* to penetrate into the pulp chamber underlines the clinical problem of microleakage, which cannot be predictably controlled with currently marketed composite materials (Rosin et al. 2002). When the crown of this tooth was exposed to basic fuchsin dye for 24 h, a clear marginal gap was visible (Fig. 3b). Under this aspect, the ideal choice of materials to rebuild the coronal structure of teeth during and after root canal treatment is a field that should be investigated in future experiments.

Conclusions

Under the experimental conditions presented in this communication, the following observations were made:

Leakage of viable *E. faecalis* from a food source into the pulp chamber occurred in a laboratory setting. 4 mm Cavit W prevented *E. faecalis* leakage under simulated mastication better than the 2 mm application of this material. A composite resin material could not predictably prevent leakage.

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