# Impact of Growth Conditions on Susceptibility of Five Microbial Species to Alkaline Stress

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## Abstract

The effects of different growth conditions on the susceptibility of five taxa to alkaline stress were investigated. *Enterococcus faecalis* ATCC 29212, *Streptococcus sobrinus* OMZ 176, *Candida albicans* ATCC 90028, *Actinomyces naeslundii* ATCC 12104, and *Fusobacterium nucleatum* ATCC 10953 were grown as planktonic cells, allowed to adhere to dentin for 24 hours, grown as monospecies or multispecies biofilms on dentin under anaerobic conditions with a serum-enriched nutrient supply at 37°C for 5 days. In addition, suspended biofilm microorganisms and 5-day old planktonic multispecies cultures were used. Microbial recovery upon direct exposure to saturated calcium hydroxide solution (pH 12.5) for 10 and 100 minutes was compared with control exposure to physiologic saline. Planktonic microorganisms were most susceptible; only *E. faecalis* and *C. albicans* survived in saturated solution for 10 minutes, the latter also for 100 minutes. Dentin adhesion was the major factor in improving the resistance of *E. faecalis* and *A. naeslundii* to calcium hydroxide, whereas the multispecies context in a biofilm was the major factor in promoting resistance of *S. sobrinus* to the disinfectant. In contrast, the *C. albicans* response to calcium hydroxide was not influenced by the growth condition. Adherence to dentin and interspecies interactions in a biofilm appear to differentially affect the sensitivity of microbial species to calcium hydroxide. *(J Endod 2008;34:579–582)*



## Key Words

Biofilm, calcium hydroxide, *Candida albicans*, dentin disinfection, *Enterococcus faecalis*, root canal


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Microorganisms commonly isolated from root canal infections are susceptible in planktonic suspensions to alkaline stress caused by calcium hydroxide in vitro (1). Even the most resistant species such as *Enterococcus faecalis* and *Candida albicans* survive only limited periods of time in the high pH of 12.5 of a saturated aqueous calcium hydroxide solution (1, 2). However, experimental eradication of *E. faecalis* from infected bovine dentin blocks has shown to be difficult (3). Most likely because of factors related to experimental infection depth, complete disinfection of extracted human premolars from *E. faecalis* by a calcium hydroxide dressing has recently been reported (4). On the other hand, a review on clinical trials has suggested limited efficacy of calcium hydroxide in the eradication of microorganisms from infected root canals after instrumentation and irrigation with a sodium hypochlorite solution (5).

Because of the number of possible confounding factors associated with clinical trials, in vitro studies are essential for the development of efficient treatment strategies in root canal and dentin disinfection. During the past decades, a wide variety of different methodologies have been introduced, and the obtained outcomes have sometimes been interpreted carelessly. Particularly, clinical conclusions based on in vitro data must be considered with care. Infected root canal systems usually contain several different cultivable taxa (6). Between microbes that can potentially invade the pulpless root canal, both positive and negative interactions have been observed. In this context, understanding the growth of individual microbial species grown in different model systems appears to be important as well as the impact of these conditions on survival of the microbes when they are exposed to an antiseptic (7). However, data on direct methodological comparisons in the endodontic literature are scarce.

The aim of this study was to compare the survival of five microbial species associated with endodontic infections under different experimental conditions. Upon an alkaline challenge induced by direct exposure to a calcium hydroxide solution, the recovery of microbiota was compared between models using single species in their planktonic form, single species adhered to dentin disks, and an established dentin biofilm comprising all five species.

## Materials and Methods

### Preparation of Dentin Disks

Standardized bovine dentin disks were prepared as follows: cylinders with a diameter of 7 mm were cut from the roots of extracted bovine front teeth using a trephine bur. Subsequently, a disk with a thickness of 0.8 mm was cut from the dentin section of the cylinder using a saw microtome (SP 1600; Leica, Wetzlar, Germany). The surface of the disk facing the outer root section was marked with a pencil. The disks were autoclaved in 121°C for 15 minutes. Disks were stored in sterile saline solution at 5°C until further usage.

### Microorganisms

The following microorganisms were used in this study: *Actinomyces naeslundii* American Type Culture Collection (ATCC) 12104, *Fusobacterium nucleatum* ATCC 10953, *Streptococcus sobrinus* OMZ 176, *E. faecalis* ATCC 29212, and *C. albicans* ATTC 90028.

**TABLE 1.** Maintenance of Microorganisms before the Experiments

| Species | Strain | Broth | Agar |
|---|---|---|---|
| *A. naeslundii* | ATCC12104 | Thioglycolate bouillon (Oxoid GmbH, Wesel, Germany) | Schaedler blood agar with Vit. K and 5% sheep blood (Becton Dickinson GmbH, Heidelberg, Germany) |
| *F. nucleatum* | ATCC10953 | Thioglycolate bouillon | Schaedler blood agar with Vit. K and 5% sheep blood |
| *S. sobrinus* | OMZ176 | Thioglycolate bouillon | Schaedler blood agar with Vit. K and 5% sheep blood |
| *E. faecalis* | ATCC29212 | Tryptic soy broth (Oxoid) | Tryptic soy agar (Oxoid) |
| *C. albicans* | ATCC90028 | Tryptic soy broth | Tryptic soy agar |

### Planktonic Bacteria

*C. albicans* and *E. faecalis* were grown on tryptic soy agar (Difco, Detroit, MI). The other three taxa were kept on Schaedler blood agar plates (Becton Dickinson GmbH, Heidelberg, Germany). Loopfuls of agar-grown cells were inoculated into fluid medium (Table 1). Precultures (5 mL) of each species were incubated anaerobically at 37°C for 24 hours (*C. albicans*, *E. faecalis*, and *S. sobrinus*) and 72 hours (*F. nucleatum* and *A. naeslundii*). Subsequently, microbial suspensions were washed and resuspended in saline. Cultures were adjusted to an optical density at 550 nm ($OD_{550}$) of 1.000 ± 0.050. The purity of the cultures was controlled by Gram-stained preparations and examination under a light microscope.

### Adherent Microorganisms

Microorganisms were grown and prepared as described previously; 1.6 mL of each microbial suspension in saline was transferred into a 24-well cell culture plate (BioMérieux, Marcy l'Etoile, France) containing a bovine dentin disk with the pulpal side facing up and incubated anaerobically at 37°C for 24 hours. Subsequently, the disks were gently dipped three times in sterile saline in order to remove loosely adherent cells.

### Monospecies Biofilm

Single strains were allowed to form a monospecies biofilm in a similar setup with dentin disks placed in 24-well cell culture plates. A protein film was obtained on the disks by incubating them in diluted horse serum (1/10 in saline) at 37°C for 2 hours. The serum solution was then replaced with 1.6 mL of fluid universal medium (FUM (8)) and inoculated with 100 μL of microbial suspension and incubated anaerobically at 37°C for 5 days. The culture media was refreshed after approximately 60 hours of incubation. Before this and in the end of the 5-day incubation period, the disks were gently dipped and agitated three times in sterile saline in order to remove loosely adherent cells.

### Five-Species Biofilm

The multispecies biofilm model used here is a modification of the model developed at the Institute of Oral Microbiology and Immunology in Zürich (9). Briefly, the species selection was adapted to endodontic interest by replacing *Streptococcus oralis* with *E. faecalis* ATCC 29212. *Veillonella dispar* was left out. In addition, the enrichment of FUM with glucose supplement was replaced by the enrichment with serum more relevant to the conditions in an infected root canal. Before infection, a protein film was obtained on the disks by incubation in diluted horse serum at 37°C for 2 hours. The disks in serum-enriched FUM (1.6 mL in each well of the 24-well cell culture plates, BioMérieux) were inoculated with 100 μL of the mixed microbial suspension and incubated at 37°C for 5 days. Refreshing the broth and removal of loosely adhered cells was performed as described earlier. All procedures and incubation were performed in a strict anaerobiosis using an anaerobic chamber with a gas mixture of $CO_2$ (10%), $H_2$ (10%), and $N_2$ (80%).

### Resuspended Microbiota From Multispecies Biofilms

To differentiate the effect of a three-dimensional structure of an established biofilm from stationary metabolic activity to susceptibility to calcium hydroxide, microorganisms grown in biofilms were suspended by a vigorous vortexing for 2 minutes followed by a gentle ultrasonication (20 W, 5 seconds; Vibracell, Sonics & Materials, Newtown, CT). Subsequently, the cells were washed and suspended in sterile saline as described earlier before their use.

### Coaggregates

To control microbial interactions without a solid substrate for biofilm formation, multispecies cultures in FUM (1.6 mL) were incubated anaerobically at 37°C on an orbital shaker (100 revolutions/min) for 5 days. The nutrient broth was refreshed after 60 hours of incubation. The obtained coaggregates were washed in sterile saline and suspended as described earlier before their use.

### Exposure to Calcium Hydroxide

One hundred microliters of each strain of planktonic, suspended biofilm grown, and suspended coaggregate-associated microorganisms were exposed to 1.9 mL of saturated aqueous calcium hydroxide solution (pH 12.5) (Merck, Darmstadt, Germany). Similarly, dentin disks with adherent microorganisms or biofilms were transferred to wells containing 2 mL of a saturated calcium hydroxide solution. Incubation in sterile saline was used as the positive control. After 10 and 100 minutes, disks were dipped and gently agitated three times in sterile saline. Each disk was transferred into a sterile 50-mL tube containing 1 mL of saline and vortexed vigorously for 2 minutes. This was followed by ultrasonication for 5 seconds. Serial dilutions of $10^{-1}$ to $10^{-5}$ in saline were prepared, and aliquots of 10 μL were plated onto agar plates listed in Table 2. Colonies were counted after 48 to 72 hours of incubation at 37°C under a stereomicroscope. Their identification was based on growth conditions, colony morphology and cellular characteristics after Gram's staining (9).

### Data Presentation

The numbers of recovered microbiota are presented as $\log_{10}$ colony-forming units (CFU/mL) in test and control suspensions (planktonic growth, coaggregates, and resuspensions) or in corresponding suspensions obtained after harvesting the adherent microbiota from the dentin disks (adhered and biofilm conditions). Data are presented as means and standard deviations.

**TABLE 2.** Selective Growth Conditions Used for Quantification of Different Species in Mixed Cultures

| Agar | Incubation | Period |
|---|---|---|
| Enterococcus BAA, Oxoid | In air | 48 h |
| CAND Biggy, Oxoid | In air | 48 h |
| Mitis-Salivarius, Difco | 5% $CO_2$ in air | 48 h |
| Schaedler blood agar | Anaerobically | 72 h |

**TABLE 3.** Susceptibility of Five Microbial Species after Different Growth Conditions to Saturated Aqueous Calcium Hydroxide Solution ($log_{10}$ CFUs/mL; $n = 5$)

| Growth | Species | 10 min | | | | 100 min | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Saline | | $Ca(OH)_2$ | | Saline | | $Ca(OH)_2$ | |
| | | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| Planktonic 24 h | *E. faecalis* | 8.28 | ±0.39 | 1.26 | ±1.16 | 8.66 | ±0.50 | 0.00 | ±0.00 |
| | *C. albicans* | 6.47 | ±0.15 | 6.18 | ±0.15 | 6.28 | ±0.38 | 2.50 | ±0.43 |
| | *A. naeslundii* | 5.39 | ±0.25 | 0.00 | ±0.00 | ND[1] | ND | ND | ND |
| | *F. nucleatum* | 6.38 | ±0.21 | 0.00 | ±0.00 | ND | ND | ND | ND |
| | *S. sobrinus* | 6.85 | ±0.19 | 0.00 | ±0.00 | ND | ND | ND | ND |
| Adherent 24 h | *E. faecalis* | 6.75 | ±0.26 | 5.62 | ±0.53 | 6.55 | ±0.22 | 2.68 | ±1.50 |
| | *C. albicans* | 5.74 | ±0.26 | 5.38 | ±0.31 | 5.27 | ±0.19 | 1.77 | ±1.39 |
| | *A. naeslundii* | 3.28 | ±0.27 | 3.28 | ±0.33 | 3.68 | ±0.67 | 0.00 | ±0.00 |
| | *F. nucleatum* | 3.02 | ±0.15 | 0.00 | ±0.00 | ND | ND | ND | ND |
| | *S. sobrinus* | 5.19 | ±0.19 | 0.00 | ±0.00 | ND | ND | ND | ND |
| Adherent 5 d | *E. faecalis* | 6.71 | ±0.33 | 6.67 | ±0.27 | 6.88 | ±0.39 | 5.44 | ±0.59 |
| | *C. albicans* | 3.86 | ±0.31 | 3.14 | ±1.79 | 3.84 | ±0.25 | 1.68 | ±0.66 |
| | *A. naeslundii* | 5.33 | ±0.66 | 5.39 | ±0.74 | 5.28 | ±0.58 | 3.73 | ±0.55 |
| | *F. nucleatum* | 4.53 | ±0.38 | 0.00 | ±0.00 | ND | ND | ND | ND |
| | *S. sobrinus* | 4.41 | ±0.35 | 0.00 | ±0.00 | ND | ND | ND | ND |
| Biofilm 5 d | *E. faecalis* | 5.97 | ±0.25 | 5.74 | ±0.46 | 5.97 | ±0.22 | 5.19 | ±0.21 |
| | *C. albicans* | 2.37 | ±0.33 | 1.51 | ±0.41 | 2.37 | ±0.27 | 0.45 | ±1.00 |
| | *A. naeslundii* | 5.89 | ±1.07 | 4.21 | ±0.44 | 5.89 | ±0.60 | 3.57 | ±0.19 |
| | *F. nucleatum* | 4.01 | ±0.32 | 0.50 | ±0.70 | 4.01 | ±1.32 | 0.00 | ±0.00 |
| | *S. sobrinus* | 4.08 | ±0.17 | 2.80 | ±0.77 | 4.08 | ±0.26 | 2.92 | ±0.61 |
| Suspended Biofilm 5 d | *E. faecalis* | 5.81 | ±0.17 | 0.00 | 0.00 | 5.78 | ±0.17 | 0.00 | ±0.00 |
| | *C. albicans* | 3.04 | ±0.24 | 1.98 | ±1.14 | 2.91 | ±0.41 | 0.34 | ±0.76 |
| | *A. naeslundii* | 5.88 | ±0.16 | 1.60 | ±1.46 | 5.71 | ±0.50 | 0.00 | ±0.00 |
| | *F. nucleatum* | 2.61 | ±0.55 | 0.00 | ±0.00 | ND | ND | ND | ND |
| | *S. sobrinus* | 4.83 | ±0.23 | 0.00 | ±0.00 | ND | ND | ND | ND |
| Suspended coaggregates 5 d | *E. faecalis* | 6.55 | ±0.22 | 0.00 | ±0.00 | 6.80 | ±0.14 | 0.00 | ±0.00 |
| | *C. albicans* | 5.57 | ±0.14 | 4.88 | ±0.34 | 5.90 | ±0.50 | 3.75 | ±0.00 |
| | *A. naeslundii* | 5.01 | ±0.47 | 1.02 | ±0.67 | 4.94 | ±1.34 | 0.00 | ±0.00 |
| | *F. nucleatum* | 2.08 | ±1.37 | 0.00 | ±0.00 | ND | ND | ND | ND |
| | *S. sobrinus* | 3.94 | ±0.23 | 0.00 | ±0.00 | ND | ND | ND | ND |

ND, not done.

## Results

In the control experiments (exposure to saline), absolute and relative numbers of planktonic, adherent and biofilm-associated CFU counts showed remarkable variation (ie, not all species grew equally well under the different conditions) (Table 3). The exception was *A. naeslundii* illustrated by similar counts regardless of the environmental parameters used in this study. However, throughout the study, data variance within the respective growth condition for the species under investigation remained low.

In general, planktonic microorganisms were most susceptible to the saturated calcium hydroxide solution, which eliminated all the species but *E. faecalis* and *C. albicans* in 10 minutes; only the latter survived in low numbers for 100 minutes. When microbiota were resuspended after growing in a five-species biofilm context for 5 days, there was no apparent change in their survival upon exposure to calcium hydroxide compared with that of early stationary-phase organisms. The same was the case with 5-day cultures of coaggregates in suspension. Dentin adhesion was the major factor in improving the resistance of *E. faecalis* and *A. naeslundii* to calcium hydroxide, whereas the multispecies context in a biofilm was the major factor in promoting resistance of *S. sobrinus* to the disinfectant. Multispecies biofilm formation also improved the survival of *E. faecalis* in comparison to planktonic or adherent cells. The difference compared with adherent organisms was not obvious at 10 minutes but appeared remarkable after 100 minutes of exposure. In contrast, the *C. albicans* response to $Ca(OH)_2$ was not influenced by the growth condition (Table 3).

## Discussion

This study showed that growth conditions differentially affect the response of the five microbiota under investigation to calcium hydroxide. Dentin adhesion was the major factor in improving the resistance of *E. faecalis* ATCC 29212 and *A. naeslundii* ATCC 12104 to calcium hydroxide, whereas the multispecies context in a biofilm was the major factor in promoting resistance of *S. sobrinus* OMZ 176 to the disinfectant. In contrast, growth conditions did not affect *C. albicans* ATCC 90028 recovery.

It should be realized that the current study was designed to compare different growth conditions of microbiota associated with endodontic disease and their impact on susceptibility of individual strains to an alkaline challenge. The multispecies biofilm model used in the current study was derived from an established six-species model that is used to test disinfectants against cariogenic plaque (9, 10). The original model has been validated and proven to yield results comparable with the situation in situ when oral antiseptics were tested at their clinically applied concentration. Compared with the original model, *Veillonella dispar* and *Streptococcus oralis* were not used in the current investigation. Instead, *E. faecalis* was introduced because this species is believed to commonly survive topical antiseptics in the root canal (11). Root dentin rather than enamel disks were used to reflect the spatial situation in the root canal. As with the original model, easily accessible laboratory strains were used. This has the advantage that the current results can be repeated in any laboratory. On the other hand, clinical strains often bear virulence factors that are lost with their laboratory counterparts. Consequently, care should be exercised to extrapolate the current results to the clinical situation.

The present data regarding the increased resistance of most microbiota to an alkaline challenge in biofilm compared with the planktonic form are in line with a multitude of publications (12). On the other hand, the results obtained with *C. albicans* presented here would contradict or at least put the commonly held paradigm that "biofilm" equals resistance in perspective. In comparison to adherent bacteria, well-established biofilms have a number of additional protective properties against antimicrobial agents. These properties include a biofilm matrix, altered growth rate of biofilm organisms, and other physiologic changes caused by the biofilm mode of growth (12). It is a prerequisite for antimicrobial agents to diffuse through the biofilm matrix in order to inhibit or kill the encased cells. The extracellular polymeric substances constituting this matrix present a diffusional barrier by affecting either the rate of transport of the agents into the biofilm or they inhibit the agents directly by binding and dilution. Another proposed mechanism for biofilm resistance to antimicrobial agents is that biofilm-associated cells grow significantly more slowly than planktonic cells and, as a result, take up antimicrobial agents more slowly (13). However, as shown in the current study, not all microbial species profit from the spatial biofilm environment to a similar extent. *C. albicans*, for example, appeared to survive but not be protected in the biofilm. It is well known from clinical observations that there are both positive and negative microbial interactions in a close spatial context (14). The five species in the current model were chosen so that no complete inhibition of any taxon occurs, but, at the same time, this model might reflect the situation of microbial aggregates in the root canal in that both positive and negative interactions appear to take place between individual species.

This study compared different growth conditions of microbiota associated with endodontic disease in order to improve the general understanding of their relative importance and thus allowing proper interpretations of in vitro findings. General validation of the model for endodontic purposes requires further investigations with combinations of clinical strains. The possible future use of this model could address the impact of type and concentration of endodontic disinfectants on the survival of the individual species and strains.

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