Clinical Implications and Microbiology of Bacterial Persistence after Treatment Procedures

José F. Siqueira Jr, PhD, and Isabela N. Rôças, PhD

Abstract

Apical periodontitis is an infectious disease caused by microorganisms colonizing the root canal system. For an optimal outcome of the endodontic treatment to be achieved, bacterial populations within the root canal should be ideally eliminated or at least significantly reduced to levels that are compatible with periradicular tissue healing. If bacteria persist after chemomechanical preparation supplemented or not with an intracanal medication, there is an increased risk of adverse outcome of the endodontic treatment. Therefore, bacterial presence in the root canal at the time of filling has been shown to be a risk factor for posttreatment apical periodontitis. About 100 species/phylotypes have already been detected in postinstrumentation and/or postmedication samples, and gram-positive bacteria are the most dominant. However, it remains to be determined by longitudinal studies if any species/phylotypes persisting after treatment procedures can influence outcome. This review article discusses diverse aspects of bacterial persistence after treatment, including the microbiology, bacterial strategies to persist, the requisites for persisting bacteria to affect the outcome, and future directions of research in this field. *(J Endod 2008;34:1291–1301)*

Key Words

Endodontic microbiology, persistent infection, retreatment, secondary infection, treatment failure

From the Department of Endodontics and Molecular Microbiology, Faculty of Dentistry, Estácio de Sá University, Rio de Janeiro, Brazil.

Address requests for reprints to Dr José F. Siqueira Jr, Faculty of Dentistry, Estácio de Sá University, Av. Alfredo Baltazar da Silveira, 580/cobertura, Recreio, Rio de Janeiro, Brazil 22790–701. E-mail address: jf_siqueira@yahoo.com; siqueira@estacio.br.

0099-2399/$0 - see front matter

doi:10.1016/j.joen.2008.07.028

The influence of bacterial persistence in the root canals on treatment outcome is an important issue in endodontics because bacteria have been shown to play a major role in persistence or emergence of apical periodontitis lesions after root canal treatment (1–9). Indeed, studies have revealed that the outcome of the endodontic treatment is significantly influenced by the presence of bacteria in the root canals at the time of filling (10–14). This indicates that persisting bacteria can survive in treated canals and are able to induce or sustain periradicular tissue inflammation, underpinning the concept that the eradication of bacteria from the root canal system should be the ultimate goal of the endodontic treatment of teeth with apical periodontitis.

This review article focuses on the microbiology and clinical implications of bacterial persistence after treatment procedures. For reviews about the microbiological aspects of posttreatment apical periodontitis associated with root canal–treated teeth, the reader is referred to other articles in the literature (15–19).

Understanding Bacterial Persistence

It is important to understand some aspects related to the significance of bacteria found in posttreatment samples. In this context, one should be aware of the time that bacterial "persisters" are detected in treated canals. Studies of the bacteria occurring in the root canal after treatment approaches involve three basic conditions: (1) postinstrumentation samples (collected immediately after completion of chemomechanical procedures), (2) postmedication samples (collected immediately after the removal of interappointment dressings), and (3) postobturation samples (collected from root canal–treated teeth with associated apical periodontitis lesion at a given time, months to years after treatment).

Studies investigating bacteria remaining in the root canals after chemomechanical procedures or intracanal medication serve the purpose to disclose the species that have the potential to influence the treatment outcome (outcome into perspective). On the other hand, studies dealing with the microbiota of root canal–treated teeth evincing apical periodontitis serve to show the association of species with treatment failure because the microorganisms detected are likely to be participating in the etiology of persistent disease (outcome already established).

Even when the endodontic treatment does not succeed in completely eradicating the infection, the huge majority of bacteria are eliminated and the environment is markedly disturbed. To survive and therefore be detected in posttreatment samples, bacteria have to resist or escape intracanal disinfection procedures and rapidly adapt to the drastically altered environment caused by treatment procedures. Bacteria detected in postinstrumentation samples are remainders of the initial infection that resisted the effects of instruments and irrigants or were introduced in the root canal as a result of a breach in the aseptic chain. Whatever the source, detected bacteria are temporary "persisters" that have not yet had enough time to adapt to the new environment, which has been changed by chemomechanical procedures. Their survival and involvement with treatment outcome will be reliant on the adaptation ability. The application of an antimicrobial intracanal medication may be the "mercy killing" for remaining bacteria. Bacteria detected in postmedication samples survived both chemomechanical procedures and intracanal medication or gained entry into the root canal via leakage through the temporary restoration. Based on the time of sampling, these bacteria have had allegedly more time for adaptation to the modified environment. Bacteria found in postobturation samples of teeth indicated for retreatment because of posttreatment disease are conceivably adapted to the new environment and are remainders of a

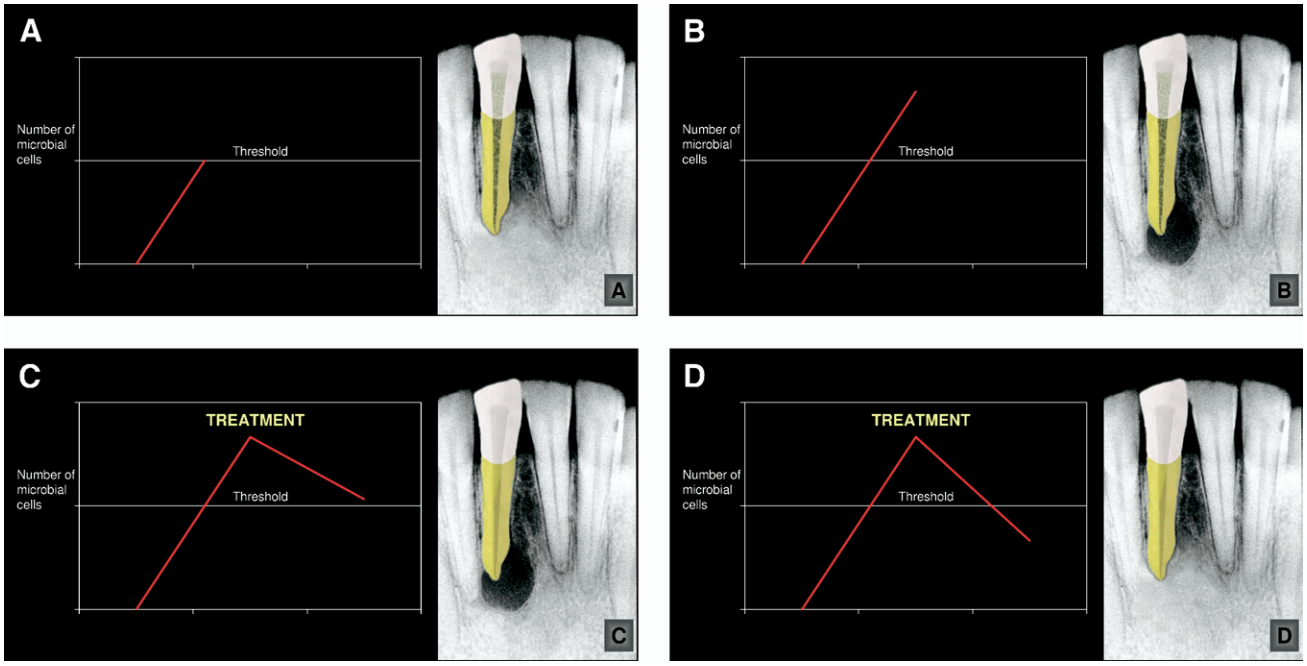

Figure 1. Microbiological goal of endodontic treatment of teeth with apical periodontitis. (*A*) Bacteria have to reach a quorum of cells sufficient to cause disease (bacterial load). Before a threshold is reached, no clinical signs and symptoms of the disease are evident. (*B*) After bacterial levels reach and exceeds that threshold, the infectious disease (apical periodontitis) is established. (*C*) If treatment procedures do not succeed in reducing bacterial levels below that threshold, the disease will persist. (*D*) Successful treatment does not necessarily sterilize the root canal but reduces bacterial populations to subcritical levels that are compatible to healing.

primary infection that resisted treatment procedures or penetrated in the root canal after filling via coronal leakage (reinfection). In these cases, failure is already established, and the bacterial species/phylotypes found in the root canals are arguably the ones to blame.

Microbiological Goals of the Endodontic Treatment

Apical periodontitis is an infectious disease caused by microorganisms colonizing the root canal system (20–23). The endodontic treatment of teeth containing irreversibly inflamed pulps is essentially a prophylactic treatment because the radicular vital pulp is usually free of infection, and the rationale is to treat in order to prevent further infection of the root canal system and consequent emergence of apical periodontitis (24). On the other hand, in cases of infected necrotic pulps or in root canal–treated teeth associated with apical periodontitis, an intraradicular infection is established, and, as a consequence, endodontic procedures should focus not only on prevention of the introduction of new microorganisms into the root canal system but also on the elimination of those located therein (25, 26). The success rate of the endodontic treatment will depend on how effective the clinician is in accomplishing these goals (27, 28).

For a better understanding of the microbiological goals of treatment of teeth with apical periodontitis, the following discussion relies on the classical observations of Theobald Smith that an infectious disease is the result of the interplay between microbial virulence and number (load) and the host defenses (29). Contextually, this concept combined with recent data on microbial community behavior, quorum-sensing mechanisms, and virulence regulation (30–32) can be applied to the understanding of the pathogenesis of apical periodontitis as an infectious disease and, consequently, can serve as a rationale for setting the goals clinicians should pursue during treatment.

It is well recognized that for any bacterial species to cause disease, they have to reach a populational density (load) that is conducive to tissue damage either caused by the bacteria themselves or by the host defense mechanisms in response to infection (33). Before a quorum of bacterial cells is reached in the infected site, no clinical signs and symptoms of the disease are apparent (Fig. 1). Conceivably, the number of cells sufficient to cause disease is inversely proportional to virulence, ie, the higher the bacterial virulence the lower the number of cells necessary to cause disease. Because endodontic infections are characterized by mixed populations of about 10 to 20 species with varying levels of virulence, it is virtually impossible to ascertain the threshold beyond which the number of cells is sufficient to induce disease. Host resistance is another important factor that impacts on disease pathogenesis. The same combination of bacterial species at the same counts may give rise to different responses in different individuals.

With this concept of bacterial load in mind, it is easy to understand the effects of treatment on the outcome of infection. Ideally, endodontic treatment procedures should sterilize the root canal (ie, eliminate all living microorganisms present in the entire root canal system). However, given the complex anatomy of the system, it is widely recognized that, with available instruments, substances, and techniques, fulfilling this goal is otherwise utopic for most cases. Therefore, the reachable goal is to reduce bacterial populations to a level below that necessary to induce or sustain disease (Fig. 1).

The challenge now is to define the bacterial levels to be achieved during treatment that are compatible with healing. Quantitative real-time polymerase chain reaction assays or fluorescence in situ hybridization using universal primers or probes, respectively, are two of the most reliable techniques to provide quantitative data from bacterial populations (34–37). However, there is no study thus far using these potent tools to evaluate the relationship between the number of bacterial cells remaining in the root canal at the time of filling and treatment outcome. Although more precise information brought about by these and other methods are still not available, it seems advisable to rely on culturing results to determine the bacterial levels that are compatible to healing. In fact, qualitative data from culture studies have been used to

establish a correlation between persistent bacteria and treatment outcome, and they have shown that occurrence of positive cultures projects poor prognosis (3, 11, 12, 14). So, in the real world, the goal of endodontic treatment is to reduce bacterial populations to levels that are not detected by culture procedures (arguably $<10^3$–10^4 cells). Reliable anaerobic culture techniques are not available for chairside tests so clinicians should be encouraged to rely on the literature to adhere to treatment protocols that are proven to predictably render root canals culture negative.

Apical periodontitis have a polymicrobial etiology, and the bacterial community profiles significantly vary from subject to subject (38–40). Differences are even more pronounced when samples from individuals living in different countries are compared (39, 41). Because of these characteristics, endodontic infections should be ideally treated by using a broad-spectrum, nonspecific antimicrobial strategy, which has the potential to reach the most possible members of the endodontic bacterial communities.

Entrenched in the privileged anatomic localization of the root canal system, bacteria are beyond the reach of the host defenses and systemically administered antibiotics. Therefore, endodontic infections can only be treated by means of professional intervention using both chemical and mechanical procedures. The main steps of endodontic treatment involved with control of the infection are represented by chemomechanical preparation and intracanal medication. Chemomechanical preparation is of paramount importance for root canal disinfection because instruments and irrigants act primarily on the main canal, which is the most voluminous area of the system and, consequently, harbors the largest number of bacterial cells. Bacterial elimination from the root canal is performed by means of the mechanical action of instruments and irrigation as well as the antibacterial effects of the irrigants. Although several irrigants have been proposed over the years, sodium hypochlorite (NaOCl) remains the most widely used (42). However, studies have revealed that chemomechanical preparation using NaOCl at different concentrations does not suffice to predictably render root canals free of cultivable bacteria; about 40% to 60% of the root canals are still positive for bacterial presence (11, 43–47). Chlorhexidine has been proposed as an alternative irrigant, but clinical studies showed that it is not superior to NaOCl with regard to antibacterial effectiveness (48, 49). Because residual bacteria can adversely affect the treatment outcome, the use of an interappointment medication has been recommended to supplement the antibacterial effects of chemomechanical procedures and eliminate persisting bacteria. Studies have shown that intracanal medication with a calcium hydroxide paste may be necessary to supplement the antibacterial effects of chemomechanical procedures and predictably render root canals free of cultivable bacteria before filling (44–47, 50, 51).

Entombment of bacteria in the canals by the root canal filling is one of the goals of the obturation phase (52). The argument that a technically well-performed root canal filling can entomb bacteria in the canal, denying them access to the periradicular tissues, is especially applicable to bacteria remaining on the root canal walls or within dentinal tubules. Bacteria remaining in the very apical part of the root canal, in apical deltas, and in lateral canals could maintain long-standing infections. Because these bacteria are in direct contact with the periradicular tissues, they have access to a sustainable source of nutrients and can maintain periradicular inflammation and impair healing. Moreover, the fact that culture-positive root canals result in a significantly worse outcome (3, 10–14) indicates that entombment does not work well, at least when the levels of bacteria in the main canal are above the detection threshold of culture. It has also been shown that the permanent root canal filling per se has a limited effect on the outcome of the endodontic treatment, even when it has been technically well performed (10). Thus, all efforts should be expended toward maximal bacterial elimination from the root canals before filling.

Persistent versus Secondary Infection as the Cause of Failure

It has not been well established whether bacteria present in root canal–treated teeth with posttreatment disease remain from previous treatment (persistent infection) or are a consequence of reinfection (secondary infection). The last 2 decades have witnessed a marked interest on the role of secondary infection resulting of coronal leakage in treated root canals as an important cause of posttreatment apical periodontitis (53, 54). However, indirect evidence seems to point to persistent infections as the most common cause of treatment failure.

Because the incidence of posttreatment disease is significantly higher in cases that showed preoperative apical periodontitis lesions (28, 55–60), it is fair to infer that persistent infections instead of secondary infections are the major cause of treatment failure. Likewise, the very high success rate of the treatment of vital (noninfected) teeth lends support to the assertion that persistent infections are the most common cause of failure in the treatment of teeth with apical periodontitis. Should secondary infections caused by coronal leakage be the most significant cause of posttreatment disease, the failure rates for the treatment of vital teeth, necrotic teeth, and even retreatment cases would be similar, but they are not (28, 55–57). The concept of secondary infection caused by coronal leakage as an important cause of failure is further put into question by the findings of a study that revealed that well-prepared and sealed root canals resisted coronal bacterial leakage even upon frank oral exposure for prolonged periods (61). However, this does not mean that the attainment of a good coronal seal is not a goal of the endodontic treatment because coronal leakage in obturated root canals can still be the cause of failure in some cases, and the clearest example seems to be those cases in which an apical periodontitis lesion was absent at the time of treatment but that appeared on follow-up radiographs.

For all these inferences to turn into definite evidence, there is a glaring need for clarification of the posttreatment fate of microorganisms detected in canals at the root canal–filling stage. The only comprehensive study dealing with this subject was an investigation in monkeys that revealed that bacteria not only can survive a permanent root canal filling for many years but also can cause persistence of apical periodontitis lesions (10). This indicates that bacteria present in the root canal at the time of filling can cause persistent infections by resisting filling procedures and materials, surviving in the changed environment, and maintaining periradicular inflammation.

Bacterial Persistence as a Risk Factor for Posttreatment Disease

Most intracanal bacteria are sensitive to standard treatment procedures. Nevertheless, some bacteria may survive treatment procedures, and their presence at the time of filling as detected by culture approaches has been recognized as a risk factor for posttreatment apical periodontitis (3, 10–14). Even though bacterial persistence may jeopardize the treatment outcome, no specific single species has been identified as a risk factor for failure. This is in agreement with the nonspecific nature of apical periodontitis etiology and apparently suggests that persistence or emergence of apical periodontitis after treatment is more dependent on the number of species remaining in the root canal than on specific bacterial taxa. However, this issue has been poorly studied and assumptions regarding the lack of bacterial specificity affecting the outcome may be mostly influenced by the dearth of consistent information.

TABLE 1. Microbial Taxa Found in the Root Canals at the Filling Stage and in Retreatment Cases as Detected in Several Separate Studies

Microorganism	Filling Stage (references)	Retreatment Cases (references)
Gram-positive bacteria		
Actinomyces naeslundii	(11, 63, 64, 92, 93, 95)	(5, 102–104)
Actinomyces odontolyticus	(11, 50, 63–65, 89, 90, 92, 93, 95)	(5, 102)
Anaerococcus prevotii	(51, 63, 64, 89, 90)	(5, 103, 105)
Eggerthela lenta	(11, 43, 89, 90)	(5, 102, 105)
Enterococcus faecalis	(11, 44, 51, 65)	(2, 3, 5, 6, 9, 67, 102–104, 106, 107)
Gemella morbillorum	(43, 63, 64, 89, 90, 108)	(5, 102, 103, 105)
Parvimonas micra	(11, 43, 44, 47, 51, 63–65, 89, 90)	(2, 3, 5, 6, 102, 103, 109)
Propionibacterium acnes	(11, 47, 50, 51, 62, 64, 89, 92, 93)	(3, 5, 102, 103, 110)
Propionibacterium propionicum	(11, 43, 64, 92, 93)	(2, 3, 5, 102, 105)
Pseudoramibacter alactolyticus	(11, 43, 44, 47, 50, 51)	(2–4, 104)
Streptococcus anginosus group	(11, 43, 47, 51, 63, 90, 92, 108)	(3, 5, 102–105, 110)
Streptococcus mitis	(46, 50, 62–64, 108)	(3, 5, 102, 103, 105, 110)
Gram-negative bacteria		
Fusobacterium nucleatum	(11, 43, 44, 46, 51, 62, 65, 89)	(2, 3, 5, 102, 104, 110)
Prevotella intermedia	(43, 51, 65, 89, 90)	(2, 5, 6, 103, 107, 111)
Fungi (yeast)		
Candida albicans	(112)	(2–4, 105, 111, 113, 114)

In cases of treatment failure, longitudinal studies evaluating bacteria at the filling stage and further at the time of retreatment have the potential to determine bacterial species/phylotypes as risk factors for posttreatment disease. Studies have shown that *Enterococcus faecalis* is the most commonly found species in root canal–treated teeth exhibiting emergent/persistent disease (2–6, 9). This might be interpreted as this species being a risk factor for persistent disease. However, *E. faecalis* has been rarely found in primary infections and not so frequent, if ever found, as a persister at the time of filling (11, 43, 44, 46, 47, 50, 51, 62–64), except in cases treated in multiple visits and/or in teeth left open for drainage (65). Recent studies have even questioned the status of *E. faecalis* as the main species involved with treatment failures (1, 66–68).

Theoretically, taxa detected at the filling stage but not at the time of retreatment may not be able to endure the conditions within obturated root canals. Likewise, taxa found only at the time of retreatment but not at the time of filling may represent secondary infections that developed by lack of a bacteria-tight coronal seal. Still following this train of thought, taxa found at both the time of filling and during retreatment of failed cases may be involved in persistent infections. Several species have been detected in both clinical conditions but in separate studies (Table 1), suggesting that they might be risk factors for poor outcomes (Fig. 2). Although all this discussion sounds logical and interesting, it is largely speculative because data belong to separate cross-sectional studies and no strong evidence can be taken in this regard. Future longitudinal studies are necessary to evaluate if the persistence of some specific species is more related to poor treatment outcome (ie, if any given species persisting in the root canal is a risk factor for posttreatment apical periodontitis).

Strategies to Persist

For bacteria to endure treatment and be detected in posttreatment samples, they must (1) resist intracanal disinfection procedures and (2) adapt to the drastically changed environment (Table 2).

Several strategies may help bacteria to resist treatment. Bacteria can adhere to the root canal walls, accumulate, and form communities organized in biofilms, which can be important for bacterial resistance to and persistence after intracanal antimicrobial procedures (69). Bacteria located in ramifications, isthmi, and other irregularities are likely to escape the effects of instruments (because of physical limitations) and irrigants (because of time constraints) used during chemomechanical procedures (70). The ability of some bacteria to penetrate dentinal tubules, sometimes to a deep extent, can also enable them to escape from the action of instruments and substances used during treatment (71, 72). Antimicrobial medicaments used in endodontics can be inactivated by dentin, tissue fluids, and organic matter (73). Some microbial species, such as *E. faecalis* and *Candida albicans*, can show resistance to calcium hydroxide (51, 74), a commonly used intracanal medicament.

In addition to escaping from treatment procedures, adaptation to the new environment is crucial for residual bacteria to cause persistent disease. A major change in the environment induced by treatment is related to a dramatic reduction in nutrient availability. The fact that the huge majority of root canal–treated teeth with posttreatment apical periodontitis have been shown to harbor an intraradicular infection (1–9) indicates that microorganisms can in someway acquire nutrients within filled root canals. Because virtually all microleakage studies have shown that no root canal–filling technique or material succeeds in promoting a fluid-tight coronal and apical seal of the root canal (75), residual microorganisms can derive nutrients from saliva (coronally seeping into the root canal) or from periradicular tissue fluids and inflammatory exudate (apically or laterally seeping into the root canal) (15). Even though most necrotic pulp tissue is removed during chemomechanical procedures, remaining bacteria can also use necrotic tissue remnants as a nutrient source. Tissue remnants can be localized in isthmi, irregularities, dentinal tubules, and lateral canals, which very often remain unaffected by instruments and irrigants (76–78). In addition, even in the main canal, some walls can remain untouched after instrumentation (76, 79, 80). Although pulp tissue remnants comprise only a temporary source of nutrients, they can maintain bacterial survival before a sustainable source of nutrients is established by apical or coronal leakage.

The fact that nutrients must exist but they are substantially reduced in amount suggests that, in order to survive, residual bacteria have to develop strategies to deal with famine. Environmental cues can regulate gene expression in bacteria, enabling them to adapt to varying environmental conditions (81). For instance, several regulatory systems play essential roles in the ability of bacteria to withstand nutrient depletion. These systems are under the control of determined genes whose transcription is activated under conditions of starvation. For instance, under conditions of nitrogen starvation, the activation of the *Ntr* gene system enables bacteria that require ammonia as a nitrogen source to scavenge even small traces of ammonia. Under high concentration of ammonia, the *Ntr* gene system is uncoupled. Under low concentrations of glucose, some bacteria can activate the catabolite repressor system, under con-

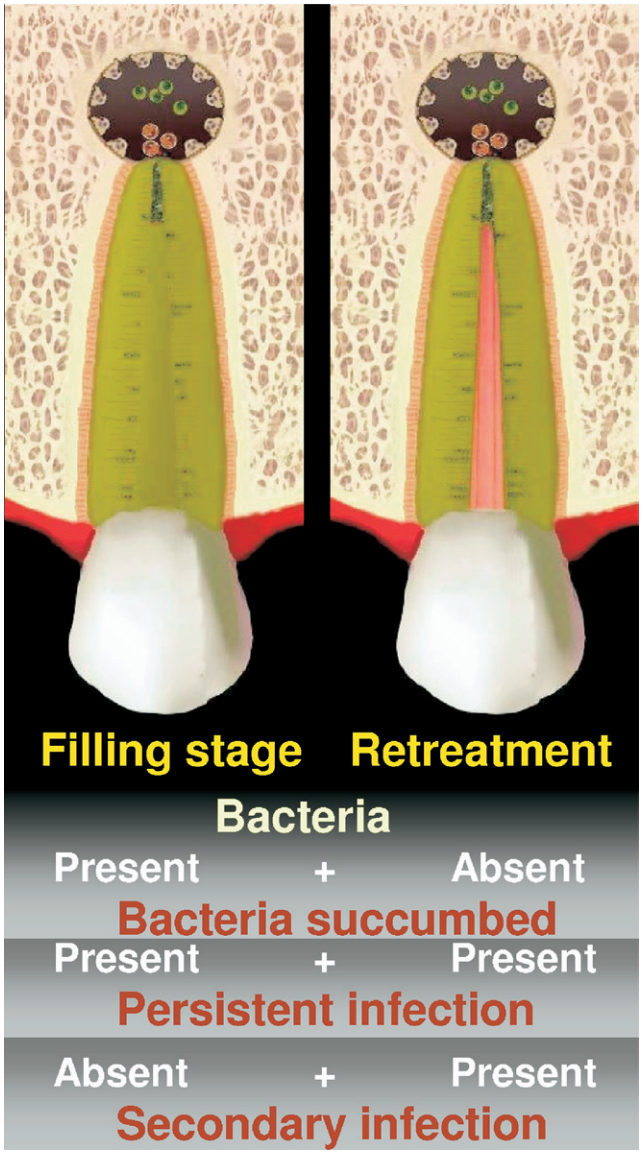

Figure 2. Interpretation of data from studies evaluating the bacterial species/phylotypes present in the canal at the time of filling (postinstrumentation or postmedication samples) or retreatment (postobturation samples). If a given taxon is found at the filling stage but not at the time of retreatment, this probably means that it succumbed in the filled root canal. If a given taxon is found both at the time of filling and at the time of retreatment, this may mean that this taxon can cause a persistent infection. If a given taxon is not detected in samples taken at the time of filling but is recovered in retreatment samples, this may mean that this taxon gained entry into the canal after filling and then is involved in a secondary infection.

trol of the genes *cya* (adenylate cyclase) and *crp* (catabolite repressor protein), which induce the synthesis of enzymes for the utilization of various other organic carbon sources. Under conditions of phosphate starvation triggered by low concentrations of inorganic phosphate, cells turn on genes for the utilization of organic phosphate compounds and for the scavenging of trace amounts of inorganic phosphate (82).

Another way to deal with changing environmental conditions is through the production of stress proteins (83). Exposure to environmental stresses may affect bacterial survival and induce accumulation of damaged or denatured proteins. In response, bacteria can induce or accelerate the synthesis of specific proteins known as stress proteins, including heat-shock proteins, which are families of highly conserved proteins whose main role is to allow microorganisms to survive under stressful conditions (84). Heat-shock proteins act as molecular chaperones in the assembly and folding of proteins and as proteases when damaged or toxic proteins have to be degraded. Several pathological functions have been associated with these proteins, including cytotoxicity that may contribute to tissue destruction (33).

It has been shown that some bacteria, such as *E. faecalis*, can enter a viable but noncultivable state (85), which is a survival mechanism adopted by many bacteria when exposed to adverse environmental conditions, including low nutrient concentrations, high salinity, and extreme pH (86). In a viable but noncultivable state, bacteria lose the ability to grow in culture media but maintain viability and pathogenicity and sometimes are able to resume division when favorable environmental conditions are restored. Figdor et al. (87) reported that *E. faecalis* has the ability to survive in environments with scarcity of nutrients and to flourish when the nutrient source is reestablished. In an *ex vivo* study, Sedgley et al. (88) showed that *E. faecalis* has the capacity to recover from a prolonged starvation state in root canal–treated teeth; when inoculated into the canals, this bacterium maintained viability for 12 months without additional nutrients. Thus, viable *E. faecalis* entombed at the time of root canal filling may provide a long-term nidus for subsequent infection.

When Residual Bacteria Influence Treatment Outcome

Bacteria persisting in the root canals after chemomechanical procedures or intracanal medication will not always maintain an infectious process. This statement is supported by the fact that some apical periodontitis lesions can heal even when bacteria were found in the canal at the filling stage (10, 11). The following are explanations for that: (1) residual bacteria may die after filling because of the toxic effects of the filling material, access denied to nutrients, or disruption of bacterial ecology; (2) they may be present in quantities and virulence that may be subcritical to sustain periradicular inflammation; or (3) they remain in a location where they have their access to the periradicular tissues denied.

Actually, bacteria that resisted intracanal procedures and are present in the canal at the filling stage can influence the outcome of the endodontic treatment provided that (1) they have the ability to withstand periods of nutrient scarcity, scavenging for low traces of nutrients and/or assuming a dormant state or a state of low metabolic activity, to prosper again when the nutrient source is reestablished; (2) they resist to treatment-induced disturbances in the ecology of bacterial community, including disruption of quorum-sensing systems, food webs/chains and genetic exchanges, and disorganization of protective biofilm structures; (3) they reach a climax population density (load) necessary to inflict damage to the host; (4) they have unrestrained access to the periradicular tissues through apical/lateral foramens or perforations; and (5) they possess virulence attributes that are expressed in the modified environment and reach enough concentrations to directly or indirectly induce damage to the periradicular tissues.

In this context, it should not be forgotten that the host resistance to infection is also an important and probably decisive counteracting factor.

Bacterial Taxa–Persisting Intracanal Procedures

Although several studies have investigated the impact of bacterial persistence on treatment outcome, not so many have consistently identified the species resisting root canal procedures (Table 3). In studies of the effectiveness of intracanal procedures, it is advisable to identify bacterial species at the baseline and after treatment so as to rule out

TABLE 2. Clinician versus Bacteria: The Bacteria Way of Deceiving Treatment

What Treatment Does	What Bacteria Have to Do to Survive
Mechanical effect: flow and backflow of irrigants	✓ Form biofilm structures firmly adhered to the canal walls; ✓ Colonize areas distant from the main canal (eg, isthmus, ramifications, and dentinal tubules)
Mechanical effect: removal by instruments	✓ Colonize areas distant from the main canal (eg, isthmus, ramifications, and dentinal tubules)
Chemical effect: irrigation	✓ Colonize areas distant from the main canal (eg, isthmus, ramifications, and dentinal tubules); ✓ Be protected by tissue remnants, dentin, serum or dead cells, all of which have the ability to inactivate or reduce the efficacy of antimicrobial agents; ✓ Be intrinsically resistant to the antimicrobial agent ✓ Form biofilm structures enclosed by a protective polysaccharide matrix
Chemical effect: interappointment medication	✓ Be protected by tissue remnants, dentin, serum or dead cells, all of which have the ability to inactivate or reduce the efficacy of antimicrobial agents; ✓ Be intrinsically resistant to the antimicrobial agent ✓ Form biofilm structures enclosed by a protective polysaccharide matrix
Ecological effect: killing of key species	✓ Adapt to the new environment, turning on survival genes and alternative metabolic pathways; ✓ Form new pairs and partnerships
Ecological effect: nutrient deprivation	✓ Adapt to the new environment, turning on survival genes and alternative metabolic pathways; ✓ Enter a viable but noncultivable state ✓ Be located in areas where nutrient sources were relatively unaffected (very apical part of the canal near the foramen, ramifications)

possible contamination during treatment, sampling, or laboratory handling of the sample. Simply detecting growth in broth or counting colonies on solid media without performing identification do not provide the same level of information as tracing identified bacterial species through a clinical case (18).

Diligent antimicrobial treatment may still fail to promote total eradication of bacteria from root canals. Persisting bacteria are either resistant or inaccessible to treatment procedures. Whatever the cause of persistence, bacterial diversity and density are substantially reduced after treatment. Root canal samples positive for bacterial growth after chemomechanical procedures followed or not by intracanal medication have been shown to harbor one to five bacterial species per case, and the number of bacterial cells usually varies from 10^2 to 10^5 per sample (11, 43, 47, 49, 50, 62) (Fig. 3).

At the time of writing this article, culture and molecular biology analysis of postinstrumentation and postmedication samples have allowed the detection of 103 bacterial and 6 fungal taxa (Table S1A in the supplemental material). Bacterial species/phylotypes detected in posttreatment samples belong to 5 phyla and 41 genera. The highest species richness has been observed for *Firmicutes* followed by *Proteobacteria* and *Actinobacteria* (Table S1A).

No single species has been significantly found to persist after treatment procedures. Gram-negative bacteria, which are common members of primary infections, are usually eliminated. Exceptions maybe include some anaerobic rods, such as *Fusobacterium nucleatum*, *Prevotella* species, and *Campylobacter rectus*, which are among the species found in postinstrumentation samples (11, 43, 48, 62, 89, 90). However, most studies on this subject have clearly revealed that, when bacteria resist treatment procedures, gram-positive bacteria are more frequently present (Table 3). Gram-positive facultatives or anaerobes often detected in these samples include streptococci (*Streptococcus mitis*, *Streptococcus gordonii*, *Streptococcus anginosus*, *Streptococcus sanguinis*, and *Streptococcus oralis*), *Parvimonas micra*, *Actinomyces* species (*Actinomyces israelii* and *Actinomyces odontolyticus*), *Propionibacterium* species (*Propionibacterium acnes* and *Propionibacterium propionicum*), *Pseudoramibacter alactolyticus,* lactobacilli (*Lactobacilli paracasei* and *Lactobacilli acidophilus*), *E. faecalis*, and *Olsenella uli* (11, 43, 46, 47, 50, 62, 63, 89–95) (Table 3). Other gram-positive bacteria, including *Bifidobacterium* species, *Eubacterium* species, and staphylococci, can also be found but in lower frequencies (11, 63, 83). This gives support to the notion that gram-positive bacteria can be more resistant to antimicrobial treatment measures and have the ability to adapt to the harsh environmental conditions in instrumented and medicated root canals.

With the recent findings showing as-yet-uncultivated bacteria as constituents of a significant proportion of the endodontic microbiota (38, 96–98), studies on the effects of intracanal antimicrobial procedures should also rely on the detection of these bacteria. A study using broad-range polymerase chain reaction and 16S rRNA gene clone library analysis investigated the bacteria persisting after chemomechanical preparation with NaOCl as an irrigant and intracanal medication with calcium hydroxide (62). Fifty-six percent of the taxa found in initial samples (baseline) were from as-yet-uncultivated bacteria. A mean of 11 taxa were detected in initial (S1) samples, 4 taxa in postinstrumentation (S2) samples, and 5 taxa in postmedication (S3) samples. The most dominant taxa in S1 samples were a novel phylotype *Solobacterium* oral clone 6Ta-2 (31% of the clones in one sample), *Bacteroidetes* oral clone X083 (37% in another sample), and *Pseudoramibacter alactolyticus* (26% in a third sample). *Streptococcus* species were detected in all posttreatment samples and were also the most dominant taxa in these samples, except for a S2 sample in which *Solobacterium* sp. oral clone K010 corresponded to 56% of the clones sequenced. Forty-two percent of the taxa found in posttreatment samples were as-yet-uncultivated bacteria. These findings suggest that previously uncharacterized bacteria may also participate in persistent endodontic infections.

Concluding Remarks

Bacteria participating in persistent infections can be identified as those present in the canal at the time of filling, although it must be recognized that many of the species found still had no sufficient time to establish a real infection and will die after filling. However, those

TABLE 3. Studies that Identified Bacteria Persisting after Intracanal Disinfection Procedures

Study	N*	Species per Case	Irrigant	Sample Taken after	Identification Method	Most Frequent Taxa (Number of Cases)	Gram-positive bacteria
Byström & Sundqvist (115)	7/15†	4.3	Saline	Chemomechanical preparation	Culture	*Peptostreptococcus anaerobius* (3) *Parvimonas micra* (3) *Lactobacillus* spp. (3) *Bacteroides* spp. (3)	21/30 (70%)
Byström & Sundqvist (43)	8/20	2.8	0.5% NaOCl	Chemomechanical preparation	Culture	*Fusobacterium* spp. (6) *Streptococcus* spp. (3) *Eubacterium brachy* (2) *Lactobacillus* spp. (2) *Porphyromonas gingivalis* (2) *Prevotella intermedia* (2)	10/22 (45%)
Byström & Sundqvist (43)	6/20	2.3	5% NaOCl	Chemomechanical preparation	Culture	*Streptococcus intermedius* (2) *Fusobacterium nucleatum* (2)	7/14 (50%)
Byström & Sundqvist (43)	3/20	2.7	5% NaOCl + EDTA	Chemomechanical preparation	Culture	*Streptococcus* spp. (2)	6/8 (75%)
Sjogren & Sundqvist (116)	7/31†	1.7	0.5% NaOCl	Chemomechanical preparation	Culture	*Fusobacterium nucleatum* (4) *Parvimonas micra* (2)	8/12 (67%)
Sjogren et al. (44)	6/12	2.3	0.5% NaOCl	Chemomechanical preparation + 10 min of Ca(OH)2	Culture	*Fusobacterium nucleatum* (3)	6/14 (43%)
Gomes et al. (90)	31	3.7	2.5% NaOCl	Chemomechanical preparation	Culture	*Streptococcus anginosus* group (14) *Parvimonas micra* (10) *Lactobacillus acidophilus* (4)	92/115 (80%)
Sjögren et al. (11)	22/55	2.3	0.5% NaOCl	Chemomechanical preparation	Culture	*Pseudoramibacter alactolyticus* (5) *Fusobacterium nucleatum* (5) *Campylobacter rectus* (4) *Parvimonas micra* (4)	28/45 (62%)
Peters et al. (89)	10/42	3.6	2% NaOCl	Chemomechanical preparation	Culture	*Actinomyces odontolyticus* (7) *Prevotella intermedia* (5) *Parvimonas micra* (5) *Eggerthella lenta* (3) *Prevotella oralis* (3)	21/36 (58%)
Peters et al. (89)	15/21	1.5	2% NaOCl	Intracanal medication–$Ca(OH)_2$	Culture	*Propionibacterium acnes* (3) *Parvimonas micra* (2) *Veillonella* spp. (2) *Bifidobacterium* spp. (2) *Capnocytophaga* spp. (2)	14/23 (61%)
Chavez de Paz et al. (94)	74	2.4	0.5% NaOCl	Intracanal medication–$Ca(OH)_2$	Culture	*Lactobacillus* spp. (40) *Streptococcus* spp. (37) *Enterococcus* spp. (26) *Propionibacterium* spp. (13)	156/177 (88%)
Kvist et al. (117)	58/94	2.1	0.5% NaOCl	Chemomechanical preparation	Culture	*Streptococcus* spp. (20) *Peptostreptococcus* spp. (17) *Prevotella* spp. (15)	84/119 (71%)
Kvist et al. (117)	16/43	1.9	0.5% NaOCl	Intracanal medication–$Ca(OH)_2$	Culture	*Staphylococcus* spp. (7) *Streptococcus* spp. (6)	27/30 (90%)
Chu et al. (63)	11/35	2.3	0.5% NaOCl	Intracanal medication–$Ca(OH)_2$	Culture	*Neisseria* spp. (4) *Staphylococcus* spp. (4) *Capnocytophaga* spp. (2) *Actinomyces* spp. (2)	15/25 (60%)
Vianna et al. (64)	8/24	1.4	2% CHX (gel)	Chemomechanical preparation	Culture	*Propionibacterium acnes* (2) *Propionibacterium propionicum* (2)	9/11 (82%)
Vianna et al. (64)	5/8	2	2% CHX (gel)	Intracanal medication–$Ca(OH)_2$	Culture	*Propionibacterium acnes* (2)	8/10 (80%)
Vianna et al. (64)	4/8	2.8	2% CHX (gel)	Intracanal medication–2% CHX (gel)	Culture	*Gemmella morbillorum* (2) *Clostridium argentinense* (2)	10/11 (91%)
Vianna et al. (64)	4/8	2.3	2% CHX (gel)	Intracanal medication–$Ca(OH)_2$/2% CHX	Culture	*Gemmella morbillorum* (2)	7/9 (78%)
Sakamoto et al. (62)	3‡	3.7	2.5% NaOCl	Chemomechanical preparation	DNA sequencing	*Streptococcus mitis* (3)	8/11 (73%)
Sakamoto et al. (62)	3‡	5	2.5% NaOCl	Intracanal medication–$Ca(OH)_2$/CPMC	DNA sequencing	*Streptococcus mitis* (3) *Streptococcus sanguinis* (2)	10/15 (67%)
Siqueira et al. (46)	5/11	1.4	2.5% NaOCl	Chemomechanical preparation	Culture/DNA sequencing	*Streptococcus* spp. (3)	5/7 (71%)
Siqueira et al. (46)	2/11	1	2.5% NaOCl	Intracanal medication–$Ca(OH)_2$	Culture/DNA sequencing	*Fusobacterium nucleatum* (1) *Lactococcus garvieae* (1)	1/2 (50%)
Siqueira et al. (47)	6/11	1.8	2.5% NaOCl	Chemomechanical preparation	Culture/DNA sequencing	*Streptococcus oralis* (2)	10/11 (91%)
Siqueira et al. (47)	1/11	1	2.5% NaOCl	Intracanal medication–$Ca(OH)_2$/CPMC	Culture/DNA sequencing	*Propionibacterium acnes* (1)	1/1 (100%)

TABLE 3. (Continued)

Study	*N**	Species per Case	Irrigant	Sample Taken after	Identification Method	Most Frequent Taxa (Number of Cases)	Gram-positive bacteria
Siqueira et al. (50)	7/13	1.7	0.12% CHX	Chemomechanical preparation	Culture/DNA sequencing	*Streptococcus mitis* biovar 2 (2)	10/12 (83%)
Siqueira et al. (50)	1/13	2	0.12% CHX	Intracanal medication–$Ca(OH)_2$/0.12% CHX	Culture/DNA sequencing	*Streptococcus mitis* biovar 2 (1) *Propionibacterium acnes* (1)	2/2 (100%)

CPMC, camphorated paramonochlorophenol; CHX, chlorhexidine.

*The Number of samples showing growth/number of samples examined.

†The number of samples showing growth after successive appointments.

‡Three samples were randomly chosen from 10 positive samples out of 15 cases treated.

that manage to survive in the new drastically modified environment can establish a persistent infection that put the treatment outcome at risk.

Bacterial persistence at the time of root canal filling has been shown to be a risk factor for posttreatment apical periodontitis. However, even though about 100 species/phylotypes have already been detected in postinstrumentation and/or postmedication samples and gram-positive bacteria are more commonly isolated/detected, it remains to be determined by longitudinal studies if any specific species/phylotypes persisting after treatment procedures can influence outcome and be considered as a risk factor.

Determination of the threshold of bacterial levels below which a favorable host response is expected can help establish a goal to focus on and has the potential do drive standardization of treatment protocols. In order words, the best treatment protocols are those that reduce bacterial counts to levels below a known threshold. For

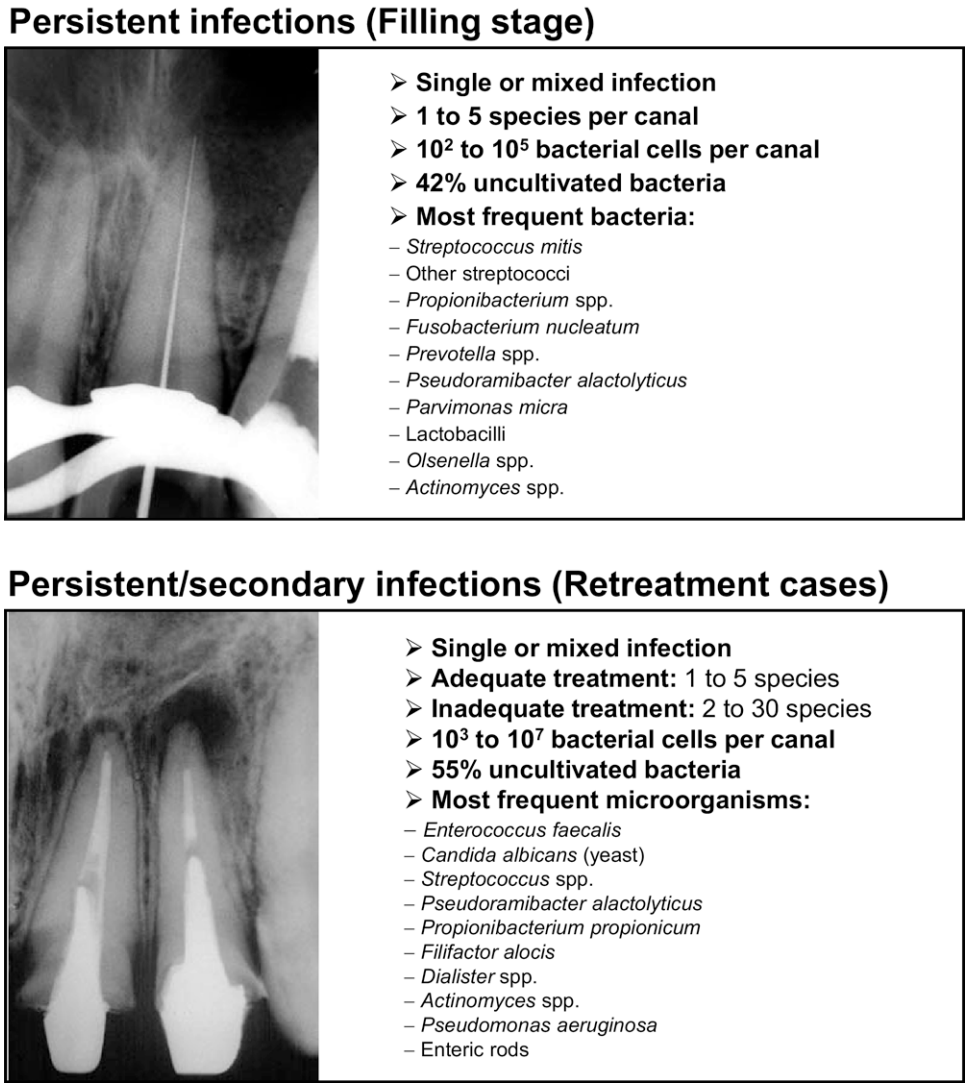

Figure 3. The main characteristics of the microbiology of samples taken at the filling stage (postinstrumentation or postmedication samples) as compared with root canal—treated teeth with posttreatment disease (postobturation samples).

want of a more reliable approach, results from culture studies are recommended as surrogate endpoints for long-term clinical outcome studies (99, 100), despite the well-recognized limitations of culturing methods (101).

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TABLE S1A. Microorganisms Detected in Post-instrumentation and/or Post-medication Samples (Filling Stage Samples) by Culture and Molecular Biology Methods

Microorganisms*	Filling Stage Samples	
	Molecular Biology Studies	Culture Studies
Bacteria		
Actinobacteria		
1. *Actinomyces gerencseriae*	(1)	
2. *Actinomyces israelii*	(1)	(2–8)
3. *Actinomyces meyeri*	(1)	(2, 3, 6–9)
4. *Actinomyces naeslundii*	(1)	(2, 3, 5, 6, 8)
5. *Actinomyces odontolyticus*	(1)	(2, 3, 5–11)
6. *Actinomyces urogenitalis*		(10)
7. *Actinomyces viscosus* (*A. naeslundii* genospecies II)	(1)	(8, 12)
8. *Bifidobacterium breve*		(2, 3)
9. *Bifidobacterium dentium*		(2–4)
10. *Bifidobacterium longum*		(2, 3)
11. *Cellulomonas parahominis*		(13)
12. *Collinsella aerofaciens*		(7)
13. *Eggerthella lenta*		(5, 7, 9, 14)
14. *Olsenella uli*		(2, 3)
15. *Propionibacterium acnes*	(15)	(2–5, 8–10, 13)
16. *Propionibacterium granulosum*		(10)
17. *Propionibacterium propionicum*		(2, 3, 5, 8, 14)
18. *Rothia* oral clone BP1-65	(15)	
19. *Rothia* oral clone BP1-71	(15)	
Bacteroidetes		
20. *Bacteroides fragilis*		(9)
21. *Bacteroides ureolyticus*		(8, 9)
22. *Capnocytophaga ochracea*		(14)
23. *Flavobacteriaceae* genomospecies C1		(15)
24. *Porphyromonas gingivalis*		(11, 14)
25. *Prevotella buccae*		(5–7, 12)
26. *Prevotella corporis*		(6)
27. *Prevotella denticola*		(6, 12)
28. *Prevotella intermedia*		(4, 7, 9, 11, 14)
29. *Prevotella loescheii*		(6)
30. *Prevotella melaninogenica*		(6, 7)
31. *Prevotella nigrescens*		(11)
32. *Prevotella* oral clone GU027	(15)	
33. *Prevotella* oral clone FM005		(10)
34. *Prevotella oralis*		(5, 9, 14)
35. *Prevotella shahii*	(15)	
Firmicutes		
36. *Aerococcus viridans*		(8)
37. *Anaerococcus prevotii*		(4, 6–9)
38. *Clostridium argentinense*		(8)
39. *Clostridium subterminale*		(6, 7)
40. *Eggerthella lenta*		(5, 7, 9, 14)
41. *Enterococcus faecalis*		(4, 5, 11, 12)
42. *Eubacterium brachy*		(14)
43. *Eubacterium limosum*		(2, 3, 9)
44. *Eubacterium nodatum*		(2, 3, 5)
45. *Gemella morbillorum*		(6–9, 14, 16)
46. *Lactobacillus acidophilus*		(2, 3, 6–8)
47. *Lactobacillus casei*		(2, 3)
48. *Lactobacillus catenaformis*		(4, 12)
49. *Lactobacillus crispatus*		(2, 3)
50. *Lactobacillus curvata*		(2, 3)
51. *Lactobacillus delbrueckii* ss lactis		(2, 3)
52. *Lactobacillus paracasei*		(2, 3, 16)
53. *Lactobacillus plantarum*		(2, 3, 7)
54. *Lactobacillus rhamnosus*		(2, 3)
55. *Lactobacillus salivarius*		(2–4)
56. *Lactobacillus garviae*		(17)
57. *Mogibacterium timidum*		(4, 5, 14)
58. *Parvimonas micra*		(4–9, 11–14)
59. *Peptostreptococcus anaerobius*		(4–6, 9, 14)
60. *Pseudoramibacter alactolyticus*		(4, 5, 10, 12–14)
61. *Ruminococcus productus*	(18)	
62. *Solobacterium* oral clone K010	(15)	
63. *Staphylococcus aureus*	(15)	(6, 10, 13, 17)
64. *Staphylococcus epidermidis*		(17, 19)
65. *Staphylococcus xylosus*		(19)
66. *Streptococcus acidominimus*		(6)

TABLE S1A. (Continued)

Microorganisms*	Filling Stage Samples	
	Molecular Biology Studies	Culture Studies
67. *Streptococcus anginosus*		(2, 7, 13, 16)
68. *Streptococcus constellatus*		(4–7, 13)
69. *Streptococcus cristatus*	(15)	
70. *Streptococcus gordonii*		(2, 17)
71. *Streptococcus intermedius*		(2, 6, 14, 16)
72. *Streptococcus mitis*	(15)	(6, 8, 10, 16, 17)
73. *Streptococcus mutans*		(2, 7, 14, 16)
74. *Streptococcus* oral clone ASCF07	(15)	
75. *Streptococcus oralis*		(2, 6, 7, 9, 10, 13, 17)
76. *Streptococcus parasanguinis*	(15)	(2, 13)
77. *Streptococcus salivarius*	(15)	(16)
78. *Streptococcus sanguinis*	(15)	(7, 10, 14)
79. *Veillonella dispar/Veillonella atypica*		(6)
80. *Veillonella parvula*	(15)	
Fusobacteria		
81. *Fusobacterium necrogenes*		(9)
82. *Fusobacterium necrophorum*		(9)
83. *Fusobacterium nucleatum*	(15)	(4, 5, 9, 11, 12, 14, 17)
Proteobacteria		
84. *Acinetobacter junii*	(15)	
85. *Aggregatibacter actinomycetemcomitans*		(6)
86. *Campylobacter gracilis*		(5, 6)
87. *Campylobacter rectus*		(4, 5, 11, 12, 14)
88. *Eikenella corrodens*		(6)
89. *Enterobacter cloacae*		(11)
90. *Enterobacter sakazakii*		(11)
91. *Kingella denitrificans*		(6)
92. *Kingella kingae*		(6)
93. *Klebsiella oxytoca*		(11)
94. *Neisseria lactamica*		(6)
95. *Neisseria mucosa*		(6)
96. *Neisseria sicca*		(6, 17)
97. *Neisseria subflava*		(8)
98. *Neisseria* oral clone BP2-72	(15)	
99. *Pantoea agglomerans*		(11)
100. *Pseudomonas aeruginosa*		(11, 20)
101. *Suttonella indologenes*		(6)
102. Uncultured *Lautropia* sp. clone 2.15	(15)	
103. Uncultured beta proteobacterium clone FAC20		(10)
Fungi		
1. *Candida albicans*		(21)
2. *Candida glabrata*		(21)
3. *Candida guilliermondii*		(16, 21)
4. *Candida inconspicua*		(21)
5. *Candida parapsilosis*		(16)
6. *Geotrichum candidum*		(21)

*Species names are updated according to the DSMZ Bacterial Nomenclature website and the International Journal of Systematic and Evolutionary Microbiology

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