Regenerative endodontic therapy (RET) is a relatively recent treatment modality because it was introduced only in the last 2 decades. Earlier attempts to revitalize the pulp space, even in mature cases, had concluded that this might be feasible in pulpitis or recently devitalized cases, but not in cases with established bacterial infection. In this regard, it was also established that spontaneous revascularization of the dental pulp tissue and continued root development after avulsion of immature teeth occur in about one-third of all cases. Thus, the emphasis from the start of RET-related research has been on enhancing the disinfection protocols in cases with infections to achieve a level that is consistent with growth of vital tissue to promote mineralization and root development. However, the concern with these disinfection protocols has been the degree to which they interfere with survival of key cellular and molecular promoters of the regenerative process and disruption of the dentinal wall structure and protein content.

When considering the regenerative potential of the dental pulp, the environment within the immature tooth with necrotic pulp has several unique features that warrant special consideration. Most important is the limited space for new vascular development, which further decreases as the tooth development proceeds and the pulp space becomes restricted in size. The increasing occlusal forces during function of the developing tooth as the patient grows older necessitate a reasonable degree of mineralization to strengthen the dentinal walls, and therefore, a tooth may function normally for a while with thin dentinal walls but may fracture as the child matures. Finally, the presence of the bacterial biofilm adherent to the surface of dentin and infiltrating the large tubules in young individuals creates a formidable challenge for the growth of new connective tissue to promote revascularization/revitalization.

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interchange of these variables within the small pulp space presents technical and biological difficulties to the clinician who is attempting to offer the patient the most predictable options for treatment. In addition, the clinician has to weigh the option of RET against apexification with a tricalcium silicate apical plug, which also has a reasonably good prognosis at least in the short and intermediate terms.10–12

Molecular microbiology provides unique tools to explore the true interaction of microbial and host factors in the context of RET. It is well-recognized that the root canal cannot be sterilized before the regenerative procedure. Nevertheless, specific antimicrobial reagents and methodologies may reduce the microbial irritants to levels that would promote apical healing, permit the formation of a viable mineralizing tissue, and allow the success of clinical procedures to be more predictable.

**ETIOLOGY AND MICROBIAL SOURCES**

Pulp necrosis in immature teeth occurs primarily because of caries, traumatic injuries, or congenital anomalies such as dens evaginatus. These conditions may occur in the period that follows tooth eruption, before root-end closure, thereby interfering with complete maturation of the tooth. Moreover, even in mature teeth and in adults, pulp necrosis may result in apical root resorption that results in widening of the apical foramen and an open apex. This makes traditional nonsurgical root canal treatment less preferred to apexification or even RET in these advanced cases.13,14 In addition, teeth may be devitalized because of microbial leakage under restorative materials or rarely in relation to excessive orthodontic forces or external invasive root resorption. The pulp is more likely to degenerate in response to trauma in teeth that have a mature compared with immature apex such as from trauma15, presumably because of reduced vascularity in mature cases. In fact, avulsed teeth with immature apex have about 30% chance for the pulp to spontaneously revascularize after replantation, whereas mature teeth always have pulp necrosis in these cases.16 In cases with mature apical foramen, nonsurgical root canal treatment is still the preferred management approach.

It is well-recognized now that endodontic microflora consists of hundreds of taxa of bacteria, viruses, and fungi. Bacteria are the main (and most studied) etiologic factor identified for pulp and periapical pathosis. The source of bacterial infection is important to recognize, because it sheds some light on the type of infection and optimal strategies for managing it. In addition, identifying the source of bacteria may help in strategies to prevent future invasion of the developing tissues after regenerative procedures. When there is a physical communication or close proximity between the oral cavity and the pulp space, such as a pulp exposure, the source of pulpal microbiota is considered to be a subset of oral, possibly subgingival microbiota.16,17 However, there has always been some controversy as to the origin of bacteria in traumatic cases with no pulp approximation18, especially that there is a similar profile that endodontic and periodontal microbiota has in general, regardless of communication19. A recent study of the microbiota identified in the pulp of teeth with normal pulp and no communication with the oral cavity or traumatic history has addressed this question19. In this study, pristine, virgin teeth that were to undergo extraction for orthodontic reasons were isolated, and their normal pulp was sampled for bacteria under rigorous methodological aseptic protocols. External plaque and saliva samples were also obtained. The samples were analyzed and sequenced by using next-generation sequencing (NGS) protocols of the 16S rRNA gene. All 10 cases revealed a plethora of bacterial taxa, most notably, members of the genera Ralstonia, Actinetobacter, and Staphylococcus, which predominated in the specimens, with minor abundances of *Burkholderia*, *Micrococcus*, *Corynebacterium*, *Micrococcaceae*, *Enterobacteriaceae*, and *Rhizobiales*, which were present in every sample. These bacteria are not typically abundant in endodontic infections because they represent sources from soil, food, tap water, and other environmental sources. However, these taxa had comparable relative abundance in samples from plaque of the same patients. This indicated that rather than a systemic origin, these bacteria may gain access to the pulp through the local blood supply from the periapical sites. They may have also reached the pulp through dentinal tubules wherever cementum is lacking, cracks in enamel, or lateral canals.

In addition to oral bacteria, systemic sources may also be involved. Circulating bacteria that could be from oral or non-oral sources may preferentially reach the site of an inflamed or degenerating pulp and co-aggregate in this location. Some older studies have provided some support for this concept20,21, whereas others did not find this to be true22. When examining this literature, it appears that the degree to which systemic bacteria may populate and contribute to pulp space infection may depend on the presence of intact circulation at some time during the active bacteremia state for the bacteria to reach its destination, i.e., this process does not take place because of diffusion of bacteria into empty space as was originally thought, but through the vascular, yet compromised blood supply.

**MICROFLORA OF ENDODONTIC INFECTIONS IN IMMATURE TEETH**

Few studies have examined the microbial content of immature teeth with pulp necrosis and apical periodontitis. Most of these cases are of children, who may or may not have a similar oral microbiome to adults. The oral microbiome is clearly affected by diet, oral hygiene, caries/restorative history, antibiotic usage, oral habits, and genetics. These factors may be different in children and adults, even those in the same family23–25. In addition, teeth take about 3 years on average after eruption to reach full maturation of their root. Therefore, when they develop pulp necrosis for any reason during that period, the bacterial biofilm formed may be of shorter duration than more mature biofilms formed in adults that may develop asymptptomatically for years. This is especially true for cases with traumatic injuries, in which follow-up is indicated to determine whether the pulp will survive, and treatment is initiated as soon as pathosis is clinically evident. The length of time has been shown to change the biofilm with respect to bacterial load, taxonomic composition and interactions26–28, metabolic activities29, as well as resistance to antimicrobial strategies30. Well-developed biofilms associated with large periapical lesions are particularly difficult to eliminate. It has been shown that the higher the bacterial load in endodontic infections preoperatively, the more likely for bacteria to persist and the higher the bacterial load of the residual bacteria after treatment31. In the cases with immature apex with large periapical lesion size, likely related to high bacterial load and presence of virulent species, inflammation leads to incomplete healing, even despite apparent mineralization and root development32 (Fig. 1). In addition to tissue irritation and inflammatory response, residual bacteria were recently shown to interfere with the release of growth factors such as transforming growth factor beta from dentin33.

Because of the high diversity of endodontic microflora, a large body of research has been dedicated to identifying key bacterial species or bacterial combinations that are sufficiently prevalent and/or are associated with symptoms or abscess formation. In the context of regenerative procedures, these bacterial taxa would be considered sufficiently virulent that they may interfere with tissue growth and cause failure of
treatment. Bacteria that fit these characteristics include gram-negative anaerobes such as *Fusobacterium* ssp., *Treponema* ssp., *Prevotella* ssp., *Porphyromonas* ssp., and *Tannerella* forsythia as well as gram-positive facultative organisms such as *Streptococcus* ssp., *Parvimonas* ssp., *Propionibacterium* ssp., *Actinomyces* ssp., and *Eubacterium* ssp.**54,39.** In addition, *Enterococcus* faecalis has not only been identified as a prevalent bacterial species in cases with persistent disease**50;** it appears to promote the survival of other bacteria in the endodontic environment and their resistance to treatment**41,42.** However, the presence of *E. faecalis* in the root canals has not been associated with symptoms**43, is not prevalent in the persistent apical lesions themselves**44, and tends to be associated with previously endodontically treated teeth whether or not they have apical periodontitis**45,46.**

One study examined the microbiota of teeth undergoing RET. Culturing of paper point samples from 15 cases at various stages of RET, followed by molecular identification of 10 specific endodontics pathogens, revealed the following species to be present: *Actinomyces naeslundii* (66.67%), *Porphyromonas endodontalis* (33.34%), *Parvimonas micra* (33.34%), *Fusobacterium nucleatum* (33.34%), *Porphyromonas gingivalis* (26.27%), *Prevotella intermedia* (26.27%), *Tannerella forsythia* (20%), *Filifactor alocis* (13.33%), and *Treponema denticola* (13.33%). Interestingly, the only species investigated that was found in none of the specimens was *E. faecalis*, and 2 cases had none of the bacteria of the group investigated, despite having positive bacteria by polymerase chain reaction. In this study, there was no instrumentation of the root canals, just biochemical disinfection. The various methods of disfections investigated, irrigation with 6% hypochlorite, 2% chlorhexidine, or 1.7% EDTA, or medication with triple antibiotic paste (Hoshino’s formula) or calcium hydroxide mixed with 2% chlorhexidine gel, reduced the counts of cultivable bacteria and mostly eliminated the specific taxa investigated. There were no differences among the medicaments investigated in disinfection efficacy**47 or in treatment outcomes**48.

However, and as noted previously, contemporary microbial analysis of endodontic infections has revealed a large diversity of microbial taxa, in which these specific microorganisms have a relatively low relative abundance**49,50.** Recent ongoing research using NGS protocols reveals that there are hundreds of bacterial taxa present in these infections, and that endodontic treatment procedures exert selective pressures to reduce bacterial load as well as the abundance of the most commonly cultivated endodontic species, but not all bacteria**51.** More specific analyses of these infections, the changes that treatment procedures cause, and the relationship of residual bacteria with treatment outcome will be published in due course. However, it is helpful at this stage to introduce some of the concepts of these types of analyses and their significance.

**MOLECULAR METHODS USED TO ANALYZE COMPLEX MICROFLORA**

Endodontic microbiology has gone through phases in its evolution that have been closely tied to technologies developed for microbial analysis. In the past few decades, the emphasis has been on cataloguing a number of microorganisms that were aerobically and anaerobically cultivated from root canals and periapical infections. There has also been an attempt to define the most likely organisms to be associated with pain and/or swelling in a manner that is consistent with Koch’s postulates, because these cause more clinically significant problems**52.** More recently and because of astounding expansion of technologies related to Sanger sequencing and NGS, it has become evident that the study of endodontic microflora should be of the characteristics of a community rather than its constituent microorganisms**53.** The reason for this is not only the presence of large numbers of different species but also the realization that as bacterial numbers in a biofilm increase, they start to express specific virulence genes, a process known as quorum sensing**54.** Layers of the biofilm develop a dormant viable but
TABLE 1 - Selected Contemporary Measures of Microbiome Analysis That Aid in the Study of Endodontics Microbial Populations

<table>
<thead>
<tr>
<th>Tool or analysis</th>
<th>Function and usage</th>
</tr>
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<tbody>
<tr>
<td>Bacterial load</td>
<td>Whereas in culture microbiology this refers to the number of colony-forming units, in molecular microbiology this refers to the relative number of bacterial 16S rRNA genes amplified using quantitative polymerase chain reaction.</td>
</tr>
<tr>
<td>OTU</td>
<td>Operational taxonomic unit, which designates a unique identity (sequence) of a microorganism identified and annotated at online databases. Similarity of sequences at the level of 97% is generally accepted to designate species identity.</td>
</tr>
<tr>
<td>Relative abundance</td>
<td>The relative proportion of an OTU compared with other OTUs identified in a sample.</td>
</tr>
<tr>
<td>Richness</td>
<td>The number of different distinguishable taxa (OTUs) present in a sample.</td>
</tr>
<tr>
<td>Alpha diversity</td>
<td>This measure describes the number of different OTUs and their relative abundance within a sample or a community.</td>
</tr>
<tr>
<td>Beta diversity</td>
<td>This measure describes the number of different OTUs and their relative abundance among different samples or communities.</td>
</tr>
<tr>
<td>LEfSe$^{61}$</td>
<td>Linear discriminant analysis effect size: an algorithm for high-dimensional biomarker discovery and explanation that identifies genomic features (genes, pathways, or taxa) characterizing the differences between 2 or more biological conditions (or classes). It emphasizes statistical significance, biological consistency, and effect relevance, allowing researchers to identify differentially abundant features that are also consistent with biologically meaningful categories.</td>
</tr>
<tr>
<td>PICRUSt$^{62}$</td>
<td>Phylogenetic Investigation of Communities by Reconstruction of Unobserved States: a bioinformatics software package designed to predict metagenome functional content from marker gene (eg. 16S rRNA) surveys and full genomes.</td>
</tr>
</tbody>
</table>

TABLE 1 (continued)

<table>
<thead>
<tr>
<th>Tool or analysis</th>
<th>Function and usage</th>
</tr>
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<tbody>
<tr>
<td>PICRUSt$^{62}$</td>
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</table>

Uncultivable state capable of resisting otherwise effective antimicrobial agents$^{55,56}$. Furthermore, the biofilm may expand into the dentinal tubules, lateral canals, and the immediate periapical area in a manner that is difficult to reach with conventional nonsurgical approaches$^{57–69}$ and also difficult to sample for study purposes. These characteristics of the bacterial community lead to evasion of host responses and intracanal treatment protocols, with resistance to the strong biocides and antibiotics that are used to disinfect the canal space. Moreover, the situation is complicated in immature teeth by the increased canal diameter apically compared with cervically, wider dentinal tubules, hesitancy to mechanically instrument the canals for fear of further weakening the tooth, and the use of low concentrations of antimicrobials to minimize toxicity to stem cells or abrogation of dentin matrix proteins$^{60}$. Some common bioinformatic tools that are currently used to analyze complex bacterial communities, which are based on analysis of the 16S rRNA gene with NGS techniques, are summarized in Table 1. In the last decade, several of these tools have been used to analyze endodontic infections. A recent critical review described the findings of 12 such studies from 2010 to 2018$^{62}$. A review of the findings shows that the top genera of bacteria identified include those that have been traditionally isolated from infected root canals (Fig. 2). However, included down that list are also a group of other non-oral genera such as Pseudomonas, Raistantia, Acinetobacter, Klebsiella, and others that have been identified in the normal pulp tissue study listed before$^{69}$ and are thought to reach the pulp from an oral source perhaps through a hematogenous or local route. It is clear from examining these data that the bacterial communities in endodontic infections are more complex than previously thought. This complexity probably plays a major role in attributes such as the development of symptoms, persistence of apical disease, and successful regenerative procedures. For example, it was recently shown that bacterial diversity in endodontic infections is significantly associated with different clinical parameters$^{54}$. Using the Shannon Diversity Index (one of the popular alpha diversity tools), the investigators showed that bacterial diversity was significantly increased in patients with systemic health problems (American Society of Anesthesiologists III versus American Society of Anesthesiologists I) and an increase in the periapical index of the apical radiolucency. They also showed that diversity was significantly reduced in symptomatic compared with asymptomatic cases, which commonly happens in diseases with biofilm dysbiosis leading to one or a few microorganisms becoming dominant, such as what happens in Clostridiodes (previously Clostridium) difficile infections after antibiotic therapy. With respect to the clinical significance of other bioinformatic tools, the exact utility and impact of these analyses are yet to be determined. However, because of the complexity of the microbiota present in these infections, they promise to offer the clinician some guidance on diagnosis of the pathologic state, selection of the appropriate personalized therapeutic approaches, and their eventual efficacy. For example, linear discriminant analysis effect size (LEfSe) analysis allows the clinician to narrow the focus on a list of fewer biologically important bacterial taxa within the whole community and to determine whether specific approaches are efficacious in eliminating these taxa$^{51}$. A recent study using LEfSe analysis showed that carious microbiota of symptomatic irreversible pulpsitis has significantly elevated levels of the phylum Firmicutes, the class Bacilli, and the genus Lactobacillus$^{62}$. Another such algorithm, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt$^{62}$), may offer an estimation of the functional attributes of different bacterial communities. This provides insight on possible expressed proteins, virulence factors, and expressed antibiotic resistance genes within the community, which may also provide insights into the best approaches, including the best antibiotics, for treatment.

ANTIMICROBIAL STRATEGIES AND THEIR Efficacy

The objective of effective root canal disinfection without interference with stem cell viability and the biofunctionality of dentin matrix has been pursued since the advent of RET$^{52,66,67}$. Current strategies and guidelines for root canal disinfection are relatively generic for cases with pulp necrosis, which range from having a mere lack of response to pulp testing with no apical changes to cases with large periapical lesions, with or without abscess formation. This wide variation in the degree to which dental tissues are infected preoperatively, which as noted previously impacts the ability to disinfect the canal, leads to the conclusion by some that...
different approaches such as calcium hydroxide or antibiotics do not affect the outcome of treatment\(^\text{68}\). For example, impressive healing at the clinical and histologic levels was recently shown in one study on traumatized teeth, in which autologous deciduous pulp stem cell grafts were used in the RET procedure\(^\text{69}\). Although the experimental group clearly showed advantages compared with the control group that had a traditional blood clot approach, the criterion for inclusion was lack of pulp response, which is common after trauma, and the radiographs presented showed minimal or no periapical lesions. This may be contrasted with cases with large periapical lesions in which traditional disinfection would achieve partial healing (Figs. 1 and 3), and additional disinfection through retreatment produced enhanced and prompt healing (Fig. 3).

Although high concentrations of biocides such as sodium hypochlorite or chlorhexidine are efficacious from an antimicrobial perspective, they have been found to cause deleterious effects on stem cells and the dentinal wall\(^\text{70-72}\) and therefore are no longer recommended for RET. A combination of low concentration hypochlorite followed by EDTA has been found to create a more biocompatible approach to the use of irrigants in these cases\(^\text{72-74}\). In addition, the use of negative pressure irrigation may minimize the toxic effects of hypochlorite on apical stem cells, which are the source of the revitalized tissue\(^\text{75,76}\).

Furthermore, although some single-visit RET cases have been reported\(^\text{77,78}\), the overwhelming majority of the literature and guidelines agree that a multi-visit approach with the use of an intervisit medicament is preferred to ensure a higher degree of disinfection. This medicament may be calcium hydroxide with or without chlorhexidine gel. Alternatively, one or more antibiotics have been proposed for use as medicaments. The following is a list of common topical antibiotics that have been reported in clinical cases or studies:

- Metronidazole, ciprofloxacin, minocycline (TAP)
- Metronidazole, ciprofloxacin (DAP)
- Metronidazole, ciprofloxacin, cefalexin
- Metronidazole, ciprofloxacin, clindamycin
- Metronidazole, ciprofloxacin, doxycycline
- Metronidazole, ciprofloxacin, amoxicillin
- Spiramycin, metronidazole
- Augmentin

The type, combination, and concentrations of antibiotics have been subjected to considerable research, much of which involved in vitro studies on efficacy, discoloration potential, and toxicity. Because of the considerable variation in the extent of initial infection, the diversity of endodontic microflora and the various other disinfection strategies (irrigant types and concentrations, degree of mechanical instrumentation, etc), it is difficult to design studies to randomize all these variables. Biocides such as calcium hydroxide appear to be effective in most cases, although one randomized trial has shown that triple antibiotic paste at the concentration of 1 mg/mL was more effective than calcium hydroxide in 2% chlorhexidine gluconate with respect to reduction of intracanal bacterial load in traditional endodontic infections\(^\text{72}\). Moreover, minocycline, which has been eliminated in many cases to prevent staining of dentin, is among the most efficacious of the antibiotics used\(^\text{19}\). Newer tetracyclines are being explored\(^\text{80}\) because of their broad spectrum of efficacy, anti-inflammatory potential, and reduced staining of dentin, allergenicity, and toxicity. Therefore, it is not clear at this time whether there is a clear indication of any one or combination of antibiotics compared with calcium hydroxide in RET cases. However, the general objectives of intracanal antimicrobials are summarized in Table 2. These goals should...
be assessed in future research to identify the optimal agents to be used.

As noted, studies show that the degree of disinfection determines the success of the treatment. The case shown in Figure 3 illustrates the challenge posed by a significant root canal infection in an immature tooth. This case also shows that after repeating the disinfection cycle and changing the medicament on retreatment, as well as eliminating the source of canal bacteria by placing an apical plug, periapical healing was rapid. Although RET could be repeated in cases like these, the patients’ parents and practitioners may opt for apexification because of the unpredictability of being able to eliminate the residual infection in these cases and the lack of chairside tools to determine antimicrobial efficacy.

It is worth noting that the recommendation for minimal or no mechanical instrumentation in these cases needs to be reconsidered. Although mechanical enlargement of the canal space is clearly not desirable because it would further weaken the tooth structure, light application of filing pressure on the root canal wall would disrupt the biofilm structure and allow root canal irrigants and medicaments to better infiltrate the biofilm. In this regard, adjuncts to irrigation such as using passive ultrasonic irrigation, irrigation using the Navitip FX brush (Ultradent Products, South Jordan, UT), and

TABLE 2 - General Objectives and Considerations for Use of Intracanal Antimicrobial Agents in RET Cases

<table>
<thead>
<tr>
<th>Broad spectrum</th>
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<td>Biocompatible and anti-inflammatory</td>
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<tr>
<td>Low minimum inhibitory concentration for key pathogens</td>
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<tr>
<td>Infiltrates biofilm</td>
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<tr>
<td>Minimal effect on dentin matrix proteins</td>
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<tr>
<td>Reduced discoloration of enamel and dentin</td>
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<tr>
<td>Substantivity and residual antibiofilm effect</td>
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<tr>
<td>Ability to be incorporated into, and remain effective in, common carriers and biodegradable scaffolds</td>
<td></td>
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<tr>
<td>Specific efficacy to patient’s own microflora</td>
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instrumentation with XP-Endo Finisher (FKG Dentaire SA, La Chaux-de-Fonds, Switzerland) or the Self-adjusting File (Redent-Nova, Ra’anan, Israel) were recently shown to aid in disinfecting large root canals\(^81\). The same recommendation was also made previously about microbial sampling of the root canal\(^82\), because paper point sampling, without biofilm disruption with a file, would most likely sample only planktonic bacteria.

**FUTURE DIRECTIONS IN RET**

Current biological research aims at enhancing the quality and predictability of RET in cases with pulp necrosis with immature and possibly mature apex. For this to occur, there must be sufficient disinfection to a threshold level that allows host immune function to assume the antimicrobial function and host cells to direct the biological healing and root maturation.

Antimicrobial strategies within the root canal environment will need to be customized to the degree and type of presenting infection. For this to occur, sufficient characterization of the microbial irritants present in infected root canals needs to be performed. Chairside diagnostic tools need to be developed to identify the optimal antimicrobials to be used and to determine the effectiveness of these agents in eliminating residual infections. This research into the personalization and customization of RET requires considerable investment of resources into these technologies, which could serve not only in cases of immature teeth but in many other endodontic cases.

In addition to enhanced antimicrobial treatment, antimicrobials could be incorporated into the scaffolds used during the revascularization/revitalization process. In fact, the antibiotics may be combined with the scaffold material, such as three-dimensional nanocomposites composed of polydioxanone and halloysite nanotubes that are designed and fabricated by electrospinning\(^83\). In general, the efficacy of antibiotics does not appear to be impeded by incorporating them into hydrogels\(^80\). Antimicrobial scaffolds can be used to allow the regenerative tissue to grow, thereby extending the antimicrobial function inside the root canal until the host tissues develop their own intracanal immune response to deal with any residual infection.

**REFERENCES**


