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REVIEW

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Understanding the microbial components of periodontal diseases and periodontal treatment-induced microbiological shifts

Ioannis Fragkioudakis[1], Marcello P. Riggio[2] and Danae Anastasia Apatzidou[1,*]

Abstract

In the mid-1960s the microbial aetiology of periodontal diseases was introduced based on classical experimental gingivitis studies . Since then, numerous studies have addressed the fundamental role that oral microbiota plays in the initiation and progression of periodontal diseases. Recent advances in laboratory identification techniques have contributed to a better understanding of the complexity of the oral microbiome in both health and disease. Modern culture-independent methods such as human oral microbial identification microarray and next-generation sequencing have been used to identify a wide variety of microbial taxa residing in the gingival sulcus and the periodontal pocket. The first theory of the 'non-specific plaque' hypothesis gave rise to the 'ecological plaque' hypothesis and more recently to the 'polymicrobial synergy and dysbiosis hypothesis'. Periodontitis is now considered to be a multimicrobial inflammatory disease in which the various bacterial species within the dental biofilm are in a dysbiotic state and this imbalance favours the establishment of chronic inflammatory conditions and ultimately the destruction of tooth-supporting tissues. Apart from the known putative periodontal pathogens, the whole biofilm community is now considered to play a role in the establishment of inflammation and the initiation and progression of periodontitis in a susceptible host. Treatment is unlikely to eliminate putative pathogens but, when it is thoroughly performed it has the potential to establish a healthy ecosystem by altering the microbial community in numbers and composition and also contribute to the maturation of the host immune response.

INTRODUCTION

Periodontitis is a chronic inflammatory disease associated with dysbiotic dental biofilms and is characterized by progressive destruction of the tooth-supporting apparatus leading to tooth loss if left untreated [1]. Its primary clinical signs are associated with attachment loss, formation of periodontal pockets, gingival swelling and bleeding and radiographic alveolar bone loss [1]. This highly prevalent non-communicable oral disease comprises a global public health problem [2] reflecting widespread social and economic inequalities [3], impairment of function, high treatment costs [4] and reduced quality of life [5]. Periodontal treatment aims to control periodontal inflammation and restore the aesthetics and function of the damaged tissues [6]. Cause-related therapy comprises patient motivation, oral-hygiene instructions and mechanical instrumentation employing non-surgical treatment techniques. Mechanical instrumentation of the exposed root surfaces can be performed by hand- and/or power-driven instruments under local anaesthesia over a varying number of treatment sessions, i.e. four consecutive scaling sessions (one for each quadrant of the dentition) at weekly or bi-weekly time intervals or one-stage full-mouth treatment visit. Modern approaches to non-surgical biofilm management and their advantages, shortcomings and controversies regarding their efficacy are comprehensively discussed in a previous review [7].

Micro-quantitative shifts in the microbial composition have been documented as a result of periodontal pocket formation [8]. Micro-organisms within the subgingival biofilm interact with each other and create a complex network of nutrient exchange, gene transfer and cell signalling, in synergy with other members of the same microbial community [9].

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Author affiliations: [1]Department of Preventive Dentistry, Periodontology and Implant Biology, Faculty of Dentistry, Aristotle University of Thessaloniki, Greece; [2]Oral Sciences Research Group, Dental School, College of Medical Veterinary and Life Sciences, University of Glasgow, UK.
***Correspondence:** Danae Anastasia Apatzidou, perioapatzidou@yahoo.gr
Keywords: periodontitis; periodontal therapy; microbiome; dysbiosis; putative periodontal pathogens; host response.
Abbreviations: EPH, ecological plaque hypothesis; FISH, fluorescence *in situ* hybridization; HOMIM, human oral microbe identification microarray; KPH, keystone pathogen hypothesis; LPS, lipopolysaccharides; NSPH, non-specific plaque hypothesis; NSPT, non-surgical periodontal therapy; PSD, polymicrobial synergy interactions and dysbiosis concept; SPH, specific plaque hypothesis; U-NSPH, updated non-specific plaque hypothesis; VAMPS, visualization and analysis of microbial population structures.

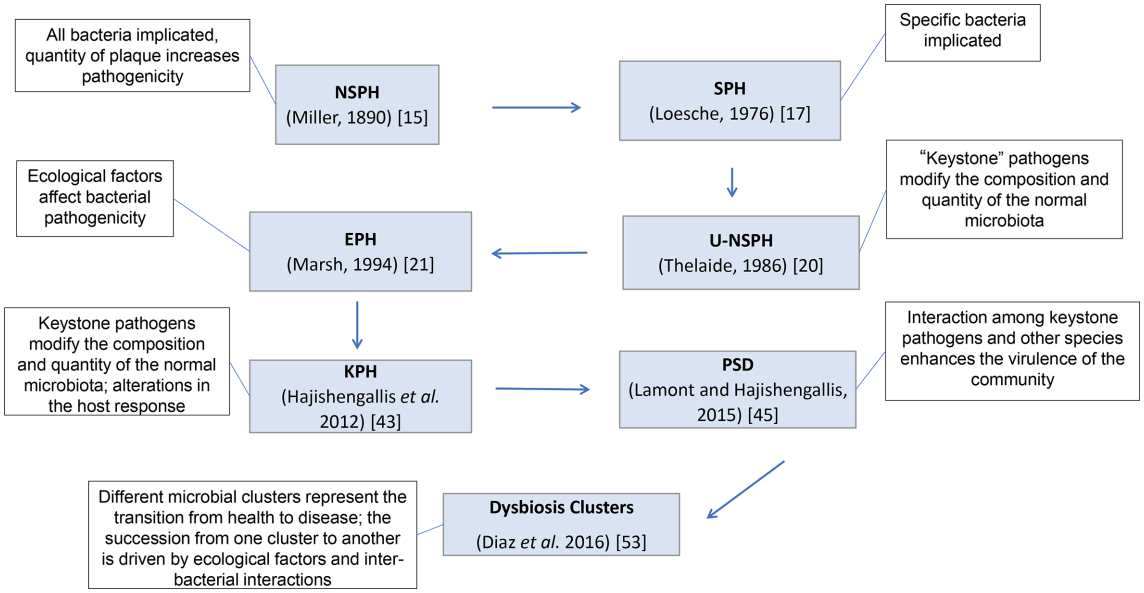

Fig. 1. Schematic diagram of the microbial concepts implicated in the aetiopathogenesis of periodontitis NSPH: non-specific plaque hypothesis; SPH: specific plaque hypothesis; U-NSPH: updated non-specific plaque hypothesis; EPH: ecological plaque hypothesis; KPH: keystone pathogen hypothesis; PSD: polymicrobial synergy interaction and dysbiosis model.

Given the complexity of subgingival multi-species microbial communities, the goal of periodontal treatment is not merely to eradicate a putative pathogen but, instead, to re-establish a harmonious co-existence of the microbial species within the periodontal niche in balance with the host. Based on this thinking, a critical question posed to the therapists is whether cause-related periodontal treatment has the potential to radically change the diseased subgingival micro-milieu having local and systemic effects on the host and the subgingival biofilms. Previous studies have proven the efficacy of non-surgical periodontal treatment (NSPT) to stimulate the host response [10, 11] and revert the microbial community into one that is more compatible with health [12, 13], highlighting the importance of periodontal therapy in establishing tissue homeostasis.

The scope of this review is to discuss the bacterial implication of periodontal diseases and also to summarize the cause-related periodontal treatment effects on the subgingival microbiota in relation to various microbiological identification techniques.

Historical perspective of the microbial aetiology of periodontal diseases

A summary of the theories implicated in the aetiopathogenesis of periodontitis and how they evolved with time is presented in Fig. 1.

Early findings in the aetiopathogenesis of periodontal diseases

The concept of how the profile of the dental biofilm affects the course of periodontitis has changed over the years and advances in the identification techniques of the implicated micro-organisms have contributed towards this end [14]. During the 'golden age' of microbiology (mid-1800s to early 1900s) putative pathogens were isolated in diseased samples and then cultivated in a medium, thereby providing evidence for the aetiology of different infectious diseases. The non-specific plaque hypothesis (NSPH) is one of the early theories that has prevailed for over a century in the aetiopathogenesis of periodontitis [15]. Based on the studies of Miller (1890) and Black (1884), this theory supported the hypothesis that the pathogenicity of the oral microbiota is determined by the quantity and not the virulence of each of the pathogenic species and that there is a specific threshold above which the host cannot cope with bacterial accumulations, resulting in inflammatory conditions [15, 16]. In the mid-1970s, advances in anaerobic culture methods led to the identification of specific bacteria in the pathogenesis of caries, giving birth to the specific plaque hypothesis (SPH) based on the concept that bacteria of the genera *Streptococcus* and *Lactobacillus* were the primary aetiologic agents in the pathogenesis of caries, and that antibiotic treatment could not halt the progression of the disease [17, 18]. The concept of SPH has also been assessed in the pathogenesis of periodontal diseases. However, the recognition that several bacteria are implicated in disease initiation and are therefore considered as putative periodontal pathogens questioned the validity of this theory [19, 20].

The indigenous microbiota associated with health was also implicated in the pathogenesis of periodontal disease, giving rise to the updated non-specific plaque hypothesis (U-NSPH) [20]. This theory, proposed by Thelaide in 1986, supported

the notion that all bacteria are putative causative agents, possessing virulence factors of varying significance. The U-NSPH recognized the role of bacterial complexity; however, this theory only partially explains the large variability noted in susceptibility to disease across individuals. In 1994, a new concept known as the ecological plaque hypothesis (EPH) was proposed [21]. Combining data from earlier theories, the EPH advocated that disease initiation is due to an imbalance in the proportion of putative periodontal pathogens within the microbial community, reflecting a state of dysbiosis in the subgingival microenvironment [21, 22]. Nevertheless, the EPH could not address the genetic predisposition to periodontal diseases [23].

Bacterial synergy and the role of the 'red complex' in periodontal pathogenesis

In the late 1990s, six bacterial complexes associated with the pathogenesis of periodontal diseases were identified to suggest that bacteria organized in complexes, namely the yellow, the green, the orange and the red complex and also the putative pathogen *Aggregatibacter actinomycetemcomitans* may act in co-operation depending on their pathogenicity [24]. The red complex is the most virulent, comprising *Porphyromonas gingivalis, Treponema denticola* and *Tannerella forsythia* [24]. The yellow complex including members of the *Streptococcus* species and the green complex comprising mostly *Capnocytophaga* species represent early colonizers of the dental plaque associated with periodontal health [25]. The members of the orange complex, comprising *Fusobacteria, Prevotella* and *Campylobacter* species represent an intermediate complex, facilitating the colonization of the red-complex species, either by providing binding sites or by creating suitable metabolic growth conditions [25]. It is noteworthy that *A. actinomycetemcomitans*, the bacterium associated with rapidly progressive disease in individuals of West African descent, does not cluster with the disease-associated red-complex members [24]. This organism is a facultative anaerobic bacterium and is considered highly pathogenic as it produces potent virulence factors such as phosphatases and leukotoxin to kill leukocytes [26].

Virulence factors of the red-complex bacteria usually include surface ligands and receptors such as lipopolysaccharides (LPSs), fimbriae, proteases (e.g. arginine- and lysine-specific cysteine proteases, a chymotrypsin-like serine protease) and metabolic end products [27]. The production of such molecules enables colonization, cell adhesion, coaggregation and cell invasion [28]. *In vitro* evidence has shown that the absence of the red complex led to enhanced streptococcal colonization during the initial stages, showing the potential of the red complex to regulate the pathogenicity of the subgingival community [29, 30]. It should be noted that members of the *Streptococcus* genus are highly abundant as oral commensals playing a key role in colonization resistance, by preventing putative pathogenic species to colonize oral sites. There is evidence that microbial organisms co-evolved with the innate defence system and developed strategies not only to overcome protective host barriers but also to manipulate the host defence mechanisms to their advantage [31]. Noteworthy, the Gram-negative bacteria, including *A. actinomycetemcomitans* and *P. gingivalis*, possess cell-surface proteins that have the ability to influence the pattern of cytokine expression by host cells [31]. The red-complex bacterium *P. gingivalis* causes inflammation and bone loss by remodelling the oral commensal microbiota and has been shown to modulate innate host defence functions that can have global effects on the oral commensal community. There is evidence that *P. gingivalis* did not induce IL-8 secretion by gingival epithelial cells and this phenomenon named 'local chemokine paralysis' strengthens the concept that this bacterium contributed to inflammation in different ways than the other oral bacteria [32].

Putative periodontal pathogens also possess the ability to invade cells [31] and both *P. gingivalis* [33] and *A. actinomycetemcomitans* [34] have been shown to invade epithelial cells where they can be found in high numbers. The relative role of the red complex and the inter-epithelial junctions expressed by gingival epithelia following microbial challenge were evaluated in a 10-species *in vitro* subgingival biofilm model or its variant model excluding the red complex [35]. Interestingly, selected desmosomal junctions in the gingiva were down-regulated irrespective of the presence of the red complex implying that this cluster of microbes might not be central in this event. These *in vitro* data indicate that the biofilm effects on the structural integrity of the epithelium might enable bacterial invasion. Following up from this, the secreted proteins (or 'secretome') of human gingival epithelial tissue challenged by similar experimental models of biofilms, indicated a down-regulation of most secreted proteins over time [36]. The authors attributed this finding to a possible attenuation of the innate host responses by the biofilm in order to evade elimination and prevail longer with the establishment of chronic inflammation. Notably in that study, the red-complex-containing biofilm was found to down-regulate the biological processes of blood coagulation and haemostasis possibly to secure the nutrients for the community. A uniform global transcriptional response of gingival fibroblasts to polymicrobial subgingival biofilm challenge is primarily associated with the innate immune response and has been found to be irrespective of the presence or absence of the red complex [37].

Collectively, the recognition of the different bacterial complexes and their virulence properties involved in the pathogenesis of periodontal disease indicated synergistic effects and possible co-dependency between different bacterial species.

Emerging theories in the aetiopathogenesis of periodontal diseases in line with advancements in microbial identification techniques

The multispecies aetiology of periodontitis, in contrast to other classical single microbe-induced diseases (e.g. tuberculosis), requires a deeper understanding of the whole spectrum of the microbial community residing within the periodontal

pocket and of the complex inter-microbial and host-microbial interactions that take place in health and disease. The introduction of bacterial 16S rRNA gene sequencing broadened the understanding of the microbiology of the oral habitat. In 2001, Paster and coworkers utilized 16S rRNA sequencing in subgingival plaque samples from subjects with various periodontal conditions and detected 374 microbial species, amongst which, 215 had never been previously identified and concluded that approximately 40% of the overall identified species were novel [38]. Kumar and coworkers analysed plaque samples of 66 adults with chronic periodontitis and 66 controls having a healthy periodontium, using 16S rRNA gene amplification, and associated five previously uncultivated species (*Atopobium rimae, Atopobium parvulum, Corynebacterium matruchotii* and two uncultivated phylotypes, clone W090 from the Deferribacteres phylum and clone BU063 from the Bacteroidetes phylum) with periodontal disease [39]. Later studies based on similar technologies identified several new phylotypes [40, 41] and at least 17 uncultivated species were found to possibly play a role in the pathogenesis of periodontal disease [42]. Similarly, high-throughput 16S rRNA gene sequencing revealed the true complexity of the periodontal microbiota and questioned the role of a single pathogen in disease initiation.

In 2012, the keystone pathogen hypothesis (KPH), was introduced (Fig. 1) and, based on studies in mice, it proposed that specific periodontal pathogens have the potential to influence and control other residents of the subgingival community, either by interacting with the commensal microbiota or by regulating the host defence mechanisms [43]. *Porphyromonas gingivalis,* albeit being in low abundance, was able to subvert the innate immunity, exerting several effects on Toll-like receptors and specific signalling pathways [44]. The KPH took into account perturbations in the host immune responses but did not consider the exact role of microbial interactions [45], as *P. gingivalis* and other members of the red complex can also be detected in healthy individuals, albeit in small numbers [46]. It should be noted that a putative pathogen considered to be a keystone pathogen is not capable to initiate disease in the absence of commensals [47]. Thus, a polymicrobial synergy interaction and dysbiosis (PSD) concept has been more recently proposed [48] (Fig. 1). The term dysbiosis is used to describe an unfavourable change in the microbial composition of an ecosystem and has been linked to several disease conditions, including inflammatory bowel disease, obesity and cancer [49]. The oral microbiota, as part of the human microbiota, evolves in parallel with the host and they both mature through the transition from childhood to adult life in a state of equilibrium. As long as this balance remains undisturbed, the oral microbiota and the host response interact in harmony, resulting in healthy and stable clinical conditions [50]. However, when an imbalance of this equilibrium occurs, the relative numbers of several putative pathogens increase, leading to an ecological breakdown that is associated with the disease. Modifying factors related to bacterial dysbiosis include lifestyle and dietary habits, compromised oral hygiene and gingival inflammation, immune system perturbations and genetic predisposition [22]. In the periodontitis model, the PSD concept suggests that a diverse microbiota colonizes the gingival crevice, and compatible species from heterotypical populations act synergistically in order to enhance their metabolic activities [48]. A keystone pathogen such as *P. gingivalis* interacts with accessory pathogens, such as *Streptococcus mitis*, leading to enhanced virulence of the entire microbial community [43]. The host immune surveillance is impaired, and dysbiotic populations are established that eventually disrupt tissue homeostasis and lead to periodontal destruction [48].

Interestingly, 16S rRNA gene sequencing of subgingingival plaque samples from patients with chronic periodontitis suggested the existence of two different bacterial clusters associated with disease [51]. Cluster-A consisted of *Fusobacterium*, *Prevotella* and *Tannerella* species, and is usually associated with gingivitis, while cluster-B with the red-complex species, other *Treponema* species and the newly emerged periodontal pathogen *Filifactor alocis*. *Filifactor alocis* is a previously unrecognized Gram-positive anaerobic rod, and its unique characteristics, virulence potential and capacity to influence the oral microbiome in community dynamics are outlined in a recent review [52]. Cluster-B was strongly associated with the severity of periodontitis. In addition, bacterial species associated with health tended to cluster in two separate groups, namely a large cluster-L, consisting mainly of *Streptococcus* and *Actinomyces species* and a smaller cluster-S consisting of *Campylobacter* and *Capnocytophaga* species [53]. A fifth intermediate cluster (named 'core species') mainly comprised *F. nucleatum* and *Bacteroides* species and was mostly associated with gingivitis, which is considered to be the transitional stage from health to disease. Combining previous data, Diaz and coworkers proposed that the transition from health to periodontal disease is determined by the existence of several microbial clusters that each represents a different stage of dysbiosis [53] (Fig. 1). The transition from one cluster to the other is dependent on parameters related to the local environment, regarding bacterial biofilm accumulation, metabolic exchanges among bacterial species and the local inflammatory stimuli. As periodontitis initiates and progresses there is an increase of the community biomass and in the diversity of the microbial taxa associated with both health and disease.

In line with previous data, higher bacterial diversity and significantly higher prevalence/relative abundance of the 'established' periodontal pathogens were found in the microbiome of periodontal patients than healthy subjects by pyrosequencing [40, 41]. This could be attributed to a richer nutritional environment subgingivally in the presence of inflammation, or a reduced immune competence in disease [54]. Interestingly, in that study, putative periodontal pathogens were also found (although at low levels) in healthy individuals implying that these organisms are not acting as exogenous pathogens. In a similar manner, a higher diversity of bacteria in disease and differences in the relative proportion of abundant phyla between health (i.e. Firmicutes phylum) and periodontitis (i.e. Bacteroeidetes phylum) suggest that

significant microbial shifts occur during the transition from periodontal health to periodontal disease [55]. In that study, the 16S rRNA metagenomic approach compared the composition of the subgingival microbiota between healthy individuals (*N*=10) and patients with severe periodontitis (*N*=10) and demonstrated that only eight genera associated with the healthy group (*Lautropia, Parvimonas, Actinomyces, Capnocytophaga, Paludibacter, Streptococcus, Haemophilus* and *Corynebacterium*) and six genera (*Porphyromonas, Treponema, Tannerella, Aggregatibacter, Peptostreptococcus* and *Filifactor*) with the diseased group [55]. Advances in identification techniques highlight the need to further investigate the role that novel bacteria may play as colonizers of periodontitis sites such as the genera *of Desulfobulbus (D. propionicus* spp.*), Mogibacterium (M. vescum* spp.*), Phocaeicola (P. abscessus* spp.*)* and *Filifactor (F. alocis* spp.). The acquired knowledge will contribute to a better understanding of periodontal pathogenesis and to the design of treatment strategies on the basis of keystone and accessory pathogens instead of pathogens and commensals [54]. Patterns of microbial communities at the genus level in subgingival samples of healthy subjects versus periodontitis patients were analysed by pooling the data of previously conducted studies using the visualization and analysis of microbial population structures (VAMPS) pipeline [56]. Specific genera (*Veillonella, Neisseria, Rothia, Corynebacterium* and *Actinomyces*) were highly prevalent (>95%) in health, while other genera (*Eubacterium, Campylobacter, Treponema* and *Tannerella*) were associated with chronic periodontitis. These data indicate that microbial analysis of chronic periodontitis samples can be correlated with severity of periodontal disease and pocket depth through specific signatures of microbial dysbiosis.

Periodontal treatment-induced microbiological shifts

Non-surgical periodontal therapy has been widely acknowledged for its efficacy to improve clinical and microbiological parameters over time [12, 57] by disturbing the subgingival biofilm and trigger the host response. Interestingly, bacteria within biofilms are in a dynamic state and detachment of cells to colonise novel sites is a principal element for their survival. However, there are a number of hurdles a micro-organism has to encounter in colonizing a periodontal pocket; the constant outflow of the gingival crevicular fluid and the presence of saliva that have anti-inflammatory and antimicrobial properties while washing out the tissues, the constant desquamation of epithelial cells, the mechanical masticatory forces, the antagonistic microbial species [6]. The microbes, which re-colonize a subgingival pocket, could be either residual micro-organisms following incomplete instrumentation or the extension of a growing and maturing supragingival plaque and there is evidence towards the former [58]. The periodontal treatment induced shifts in the subgingival ecosystem can be maintained in balance in the long-term through meticulous home-care plaque control measures and by supportive periodontal therapy that entails professional removal of the accumulated soft and hard deposits on the affected root surfaces and reiteration of oral-hygiene instructions for home care [59].

Advances in bacteria identification techniques contributed to a comprehensive evaluation of treatment effects, regarding perturbations of the microbial community and changes in re-colonization patterns. A landmark study examined the effects of treatment on bacteria succession, suggesting three distinct re-colonization patterns. A rapid reduction and slow return were noticed for the total bacterial counts and spirochaetes, while a rapid reduction accompanied by a rapid return was followed by Gram-negative anaerobic species. *Streptococcus* and *Actinomyces* species followed a different pattern of a rapid increase and slow reduction after therapy [12]. Several studies have assessed the microbiological effects of different designs of NSPT regimens regarding partial versus full-mouth treatment protocols in terms of re-colonization patterns and these findings are discussed in a comprehensive review [7]. Optimal treatment outcome is guaranteed by thorough plaque control, which ensues from highly motivated and skillful patients in oral-hygiene practices for the long-term maintenance of plaque-free periodontal tissues following careful mechanical instrumentation [6]. Interestingly, in compact protocols of mechanical instrumentation and in the absence of oral-hygiene reinforcement, the 3 month reduction in numbers of *P. gingivalis* as determined by the checkerboard DNA–DNA hybridization technique and in plaque and bleeding indices were significantly smaller compared with the group that received re-iterated oral-hygiene instructions and motivation [60]. However, these inter-group differences disappeared at 6 months following additional treatment given at 3 months. These data highlight the importance of professional removal of dental biofilm every 3 months in subjects with compromised plaque control. The microbiological effects of these treatment protocols are summarized in Table 1.

Early microbiological treatment outcomes based on culture-dependent techniques

Early studies mainly based on microscopy and culture methods addressed the effects of periodontal therapy on the bacterial composition of the periodontal pocket [12, 61–63]. In the 1970s and 1980s, microscopy was utilized as a relatively simple and inexpensive technique to detect bacteria; however, it was limited to detection of a small number of morphotypes, failing to identify a wide range of bacterial species [64]. Nevertheless, phase-contrast and dark-field microscopy studies overcame these limitations and offered valuable information regarding bacterial species associated with health and disease [65]. The treatment effects on the subgingival microbiota can be summarized as a significant reduction of the percentages of spirochaetes and motile rods and a concomitant increase in coccoid cells and *Actinomyces* species [12, 61, 62, 65–68]. These beneficial microbial changes were paralleled by improvements in the clinical parameters of moderately deep (3–4 mm) and deep (≥5 mm) periodontal pockets [66, 69].

Table 1. Periodontal treatment effects on the microbial communities determined by bacterial identification techniques

Microbiological technique	Microbiological perturbations	Principal findings
Culture [73–77]	Reductions in total bacterial counts, *Bacteroeides spp., Fusobacterium spp., P. gingivalis, P. intermedia, A. actinomycetemcomitans*	Study the timeframe of recolonisation patterns
PCR [11, 82, 85, 86]	Reductions in *P. gingivalis, T. forsythia. P. intermedia, A. actinomycetemcomitans*. Increases in *Actinomyces spp*., *Streptococcus spp*., and *Veillonella parvula*	Clinical improvements in parallel with microbiological reductions. Non-surgical periodontal therapy fails to eliminate the test putative pathogens
DNA–DNA Checkerboard Hybridisation [12, 92, 93]	Reductions in *P. gingivalis, T. forsythia. P. intermedia, A. actinomycetemcomitans*. *Increase of A. Viscosus, Actinomyces Spp*.	Most significant microbial reductions are found within the first 3 months post-treatment, however sustained up to 12 months
Next Generation Sequencing [96, 99, 100]	Reductions in previous tested putative pathogens. Reductions in the levels of novel pathogens (*i.e. F. alocis*)	Treatment disrupts inter-species connections

Culture techniques contributed to the microbiological understanding of periodontal diseases by identifying some of the key putative pathogens associated with gingivitis and periodontitis [70, 71]. Nevertheless, culture techniques are considered to be labour-intensive and are often consequently restricted to analysing limited numbers of samples and species at a time [64], and may often underestimate the true number of taxa [72]. Significant post-treatment reductions in total bacteria counts and in the proportions of specific species of the subgingival microbiota such as *Bacteroides* and *Fusobacterium* genera, *Aggregatibacter* genus (*A. actinomycetemcomitans* spp.), *Prevotella* genus (*P. intermedia* spp.) and *Porphyromonas* genus (*P. gingivalis* spp.) have been demonstrated using culture methods [73–77]. Van Winkelhoff *et al.* observed a significant reduction in *P. gingivalis* counts 2 weeks after a single course of scaling and root-planing, which was sustained for 8 weeks [61]. Similarly, Pedrazzoli *et al.* in a study employing a split-mouth design found significant reductions in the relative proportions of *Bacteroides* genus, 2 to 4 weeks following periodontal therapy (surgical and non-surgical), followed by an increase at weeks 8 to 16, though failing to reach baseline values. Simultaneously, a significant increase in *Streptococcus* species counts was found 4 weeks post-treatment [74]. Both studies demonstrated a trend towards a return of the microbiota to the initial counts after week 4 [61, 74], but without any deterioration in periodontal clinical parameters. Two classical studies investigated the effects of a single session of scaling and root-planing on the microbiota in the absence of oral-hygiene instructions and the time required for the microbial species to return to baseline values [68, 78]. There was an agreement in both studies that 42 to 60 days are required for the microbial proportions to return to baseline scores, but without concomitant clinical signs of disease recurrence.

Although early microbiological identification techniques are limited to detecting certain species, they have provided valuable information regarding the beneficial effects of NSPT on the composition of the subgingival microbial community.

DNA-based detection methods to assess treatment-induced microbiological shifts

The introduction of DNA-based methods offered the possibility to analyse several samples at a time, allowing for a rapid, less laborious and inexpensive analysis [79]. Both the PCR and the use of DNA probes do not depend on cell viability and have been proven to be more sensitive compared to culture techniques by having lower detections limit of up to 50 cells [80, 81]. Studies based on such methods have broadened the existing knowledge on treatment effects on the subgingival microbiota and revealed the inability of periodontal therapy to eliminate the putative periodontal pathogens tested [82]. The molecular microbial analyses, i.e. PCR-based methods, DNA–DNA hybridization and sequencing are independent from *in vitro* cultivation and their contribution to microbial diagnosis are outlined in a comprehensive review [83].

PCR to assess microbiological treatment outcomes

In this regard, the conventional PCR technique offers high sensitivity and specificity in bacterial detection, despite its limitation to identify targeted species only [64]. However, multiplex PCR can simultaneously detect multiple species [84] and the introduction of real-time PCR has also enabled quantitative analysis of the subgingival periodontal community [82], enabling PCR methods as valuable tools in periodontal microbial diagnosis. A benchmark study of 28 adult periodontitis subjects showed that substantial alterations in the numbers of *P. gingivalis* and *T. forsythia* occurred 1 month after treatment [11]. PCR analysis showed that the mean prevalence of *P. gingivalis, T. denticola* and *P. intermedia* is significantly reduced from 6 weeks to 6 months post-therapy and this was accompanied by significant improvements in clinical parameters [11, 82, 85]. A study of similar design of 9 months duration revealed significant decreases in levels and percentages of *P. gingivalis, T. forsythia, A. actinomycetemcomitans* and *Campylobacter rectus* at 3 and 6 months following therapy [86]. Reduction of periodontal pathogens was accompanied by a significant increase in the proportions of certain beneficial bacteria such as *Actinomyces* species, *Streptococcus* species and *Veillonella parvula* over time [86]. When real-time quantitative PCR was utilized,

a positive association was suggested between the counts of specific pathogens and the clinical signs of periodontitis [82], in contrast to other studies, which failed to unveil a clear relationship between clinical indices and microbiological findings [11].

DNA hybridization-based methods for microbiological treatment outcomes

Hybridization-based methods employed in oral microbiological research include whole-genome checkerboard DNA–DNA hybridization, reverse-capture oligonucleotide hybridization (which is a modification of the checkerboard method), fluorescence *in situ* hybridization (FISH) and DNA microarrays. Fluorescence *in situ* hybridization (FISH) and whole-cell hybridization has been used in oral microbiological research to mainly study the structure of the oral biofilm [87], although these methods have not provided information on periodontal treatment-induced microbial changes. The introduction of whole-genomic checkerboard DNA–DNA hybridization techniques allowed the recognition and quantification of up to 45 selected cultivable bacteria per assay [88]. Although initially having the disadvantage of misidentification of species due to cross-hybridization of whole-genomic DNA probes and thus having lower specificity than oligonucleotide probes, this technique enabled the qualitative and quantitative analysis of several samples at a reasonably high level of specificity (93.5%) [88, 89]. It also enabled the identification of the potential synergic interaction between bacterial species and introduced the classification of microbiota into five complexes based upon their level of pathogenicity [24, 88]. However, this method only allows the identification of previous cultivated taxa and even if it uses a large number of probes (generally no more than 45) it cannot accurately determine the diversity of the subgingival environment where more than 300 species reside [90]. An earlier study using DNA probes for *A. actinomycetemcomitans, P. gingivalis* and *P. intermedia* found significant reductions in the mean numbers of bacteria at both 1 week and 1 month post-therapy [91]. Haffajee *et al*. examined the effects of periodontal therapy in 57 subjects at 3 and 6 months post-therapy, and demonstrated a significant decrease in the levels of *T. forsynthia, P. gingivalis* and *T. denticola*, with a parallel considerable increase in the level of *A. viscosus*, associated with periodontal health [13]. A follow-up study looked into a subset of 32 patients during supportive periodontal therapy and found a significant reduction in the numbers of *T. forsythia, P. gingivalis* and *T. denticola* but an increase in *Actinomyces* species up to 12 months post-treatment [92]. Haffajee and coworkers, in an effort to understand the effects of periodontal therapy on the composition of the subgingival microbiota, suggested that NSPT, apart from decreasing the microbial burden and suppressing specific microbial species, induces significant changes in the microenvironment of the periodontal pocket (i.e. gingival crevicular fluid reduction, probing pocket depth reduction) that enable tissue homeostasis, even though re-colonization of certain bacteria might occur in the long term [93].

The 16S rRNA-based oligonucleotide checkerboard hybridization (reverse-capture oligonucleotide hybridization) method has been employed in the diagnosis of selected putative periodontal pathogens that have been cultivated or not yet cultivated [94] and modifications of this technique, named oligonucleotide microarray technology, by carrying out hybridization on glass slides led to the development of the human oral microbe identification microarray (HOMIM) to simultaneous identify numerous bacterial species, including those that have not yet been cultivated [83]. Colombo and coworkers compared the subgingival microbiota of three groups of subjects (refractory periodontitis, good responders to periodontal treatment, periodontally healthy) in an attempt to determine the poor responders based on their baseline subgingival microbial profiles [95]. Subjects with refractory periodontitis were distinguished from good responders or those with periodontal health by a significantly higher frequency of putative periodontal pathogens, speculating that an ineffective host response to infection, high load and/or virulent species might account for a modest response to treatment despite the reductions seen in putative pathogens. In a subsequent study from the same research group, the treatment effects of mechanical and antimicrobial therapy were evaluated in refractory periodontitis patients versus good responders to treatment [96]. Following treatment, the majority of putative periodontal pathogens were reduced in good responders whereas periodontal pathogens and other potential pathogenic novel species or phylotypes persisted in high prevalence in sites and/or individuals that responded poorly to treatment, necessitating the need for design of justified treatment approaches in the latter category of patients [96].

Advances in DNA sequencing: next-generation sequencing for microbiological treatment outcomes

Novel microbial identification techniques based on conserved and hypervariable regions of the 16S rRNA of a species employing open-ended methods have contributed to a better understanding of the whole microbial community by not limiting analysis to specific species [39], but rather by revealing changes that occur in the complex subgingival microbial community. Next-generation sequencing is nowadays routinely used to perform very-high-throughput genome sequencing that allows millions to trillions of observations to be made in parallel during a single run at a reasonable cost [97]. Applications and limitations of these methods are summarized in a recent review [98], while an overview of second-generation sequencing manufacturers gives an insight of the systems that are currently available in the market [97]. In this context, Kumar and coworkers assessed the microbial community shifts after NSPT using culture-independent, quantitative 16S rRNA cloning and sequencing. In their study, subgingival plaque samples were collected from 24 individuals with various periodontal conditions over a 2 year period [96]. Clinical stability was strongly correlated with microbial stability while progression of periodontal disease was associated with a less stable subgingival microflora. Regarding individual species, the levels of *Veillonella* species clone X042

was associated with health, while *F. alocis* levels were higher in advanced diseased subjects. A later study indicated that the relative abundance of the putative periodontal pathogens *P. gingivalis, T. forsythia* and *T. denticola* decreased 6 weeks after treatment, whereas *Rothia aeria, Rothia dentocariosa, Streptococcus* species and *Actinomyces* species increased in subgingival plaque samples following therapy [99]. Shi and coworkers analysed subgingival plaque samples before and after NSPT from 12 individuals using metagenomic shotgun sequencing [100]. Apart from the differences in microbial composition among diseased and treated sites, a striking finding of that study was the disruption in the co-occurrence of species from the genera *Porphyromonas, Tannerella, Mycoplasma, Filifactor, Synergistes* and *Treponema,* which are all associated with periodontal disease, thereby indicating an alteration among the intra-species connections. In addition, the richness of the microbial flora was decreased after treatment [100]. The aforementioned data justify a polymicrobial aetiology of periodontitis that questions the role of a single pathogen in disease initiation.

Deep sequencing of oral samples collected from chronic periodontitis patients demonstrated decreases in the relative abundance of subgingival plaque-specific bacteria in the salivary microbiota following periodontal therapy having significant associations with clinical improvements [101]. Next-generation sequencing was used to determine the non-surgical treatment effects on the saliva microbiome of generalized aggressive periodontitis patients [102]. Clinical improvements were not paralleled by relative abundance changes on the phylum, genus or species level, or diversity indices indicating a resilience of the salivary microbiome to treatment in this category of periodontitis patients. Interestingly, that study attributed the observed limited microbiome shifts in patients with poor treatment outcomes to a potential emergence or inefficient elimination of virulent species, or to a failure of establishment of commensal species following treatment [102]. The subgingival microbial profile of well-maintained periodontitis patients (*N*=14) having no evidence of clinical inflammation and progress of periodontitis was compared with healthy subjects (*N*=21) by sequencing the V3-V4 region of the 16S rDNA [103]. Well-maintained subjects had a more dysbiotic microbial community with more pathogenic periodontitis-associated bacteria (i.e. genera of *Leptotrichia* and *Treponema*) and less beneficial taxa (i.e. genera of *Streptococcus* and *Granulicatella*), higher diversity, more disordered structure and more host-destructive metabolism pathways than healthy individuals. These data highlight the need to closely monitor subjects who despite having no overt clinical signs of periodontitis recurrence, they require supportive periodontal therapy to maintain their microbiome in balance and thus, achieve long-term periodontal stability.

Noteworthy, open-ended high-throughput microbiome sequencing can identify many hundreds of species, however overlooking low copy number organisms, which can be key players in orchestrating the microbial community and close-ended sensitive molecular methods (i.e. quantitative-PCR) can unveil these more distinctive effects of treatment [102].

Overall, despite the major contribution of the aforementioned studies, the impact of NSPT on the host tissues should be studied not only from a microbiological viewpoint but also by looking into perturbations of the host response in line with the dysbiotic model of disease.

CONCLUSIONS

The efficacy of NSPT in terms of clinical and microbiological improvements has long been established [57, 93]. The microbial effects of NSPT, as described above, can be summarized by a decrease of motile anaerobes and spirochaetes and an increase of cocci and motile bacteria [57]. A predominantly Gram-negative anaerobic microbial community is reversed into a Gram-positive one following treatment [57]. In other words, putative periodontal pathogens decrease, giving rise to beneficial species more compatible with health. Interestingly, studies employing contemporary molecular techniques offered valuable information regarding previously uncultivated species that play a key role in periodontal pathogenesis such as *Filifactor alocis* [99]. On the other hand, *Veillonella* species oral clone X042, a Gram-negative bacterium, was the most common member of the subgingival bacterial community and was associated with periodontal health. These data suggest that the aetiopathogenesis of periodontitis appears to be more complex than initially thought and monitoring the levels of these bacteria – subgingivally and in saliva [100] – may prove clinically useful [99]. With regard to the keystone pathogen *P. gingivalis*, a number of studies have demonstrated the ability of periodontal treatment to decrease the levels of this pathogen in a relatively short period of 6 weeks post-treatment [11, 74].

The dysbiotic model of disease initiation requires the disruption of the balance among bacterial species and the establishment of a diverse bacterial community led by complex interspecies interactions in which a pathogenic microflora prevails [53]. New studies based on modern identification techniques have proved the ability of NSPT to restore the dysbiotic state in terms of bacterial diversity decrease and inter-bacterial interactions disturbance [100]. Nevertheless, it is unlikely that scaling and root-planing can eliminate all micro-organisms from a site, signifying that the presence of a suspected pathogen at a site is not indicative of disease activity [82]. It appears that the reduction of putative periodontal pathogens, namely *P. gingivalis, T. denticola* and *P. intermedia*, following subgingival mechanical instrumentation initiates shifts in the local ecosystem that lead to periodontal stability [64, 89]. Such changes may be seen for up to 2 years following treatment, although microbial re-colonization may also occur [104]. It should be emphasized that post-treatment microbial changes are closely associated with the clinical outcome as the reduction of several pathogens such as *P. gingivalis* is correlated with the reduction of probing pocket depth and bleeding indices [11, 73, 74].

In general, the main outcome of NSPT is not the eradication of specific pathogens but rather the establishment of a healthy ecosystem by altering the microbial community in numbers

and composition and contributing to the maturation of the host response. The ecosystem appears to return to one comparable to periodontal health. Thus, the main therapeutic target is to turn the clock back and return the current status to the one before disease was initiated, implying that the subgingival microbiota has to go through prolonged modifications in composition and quantities to reach the status required for disease occurrence [90, 104].

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The authors declare that there are no conflicts of interest.

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