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Microbial signatures of health, gingivitis, and periodontitis

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1 | INTRODUCTION

Subgingival microbial communities are dynamic assemblies. These complex entities harbor microorganisms from the 3 domains of life, namely Bacteria, Archaea, and Eukarya.[1] Bacteria are the most numerous and diverse component of the subgingival microbiota: to date, about 500 different species have been detected in human subgingival plaque.[2,3] Classic cultivation-based studies have reported that the composition of subgingival bacterial communities differs in health, gingivitis, and periodontitis.[3-5] In recent years, a detailed description of the composition of oral microbial communities has been made possible by high-throughput DNA sequencing. In particular, the use of phylogenetic markers, such as the 16S ribosomal RNA gene, has facilitated characterization of the oral microbiome with high taxonomic resolution and in a cost-effective manner, in a large number of subjects. Although few molecular studies have directly compared subgingival communities in health, gingivitis, and periodontitis, 16S ribosomal RNA gene-based studies seems to agree with the cultivation-based results of previous decades, in that unique microbial signatures exist for each state (reviewed in Diaz et al[6]).

Understanding the specific shifts in composition of the microbiome that are associated with each periodontal condition is important because the nature of the microbial stimuli is a determinant of whether protective or pathogenic host responses take place at the gingiva. Although periodontitis is linked to genetic and environmental determinants that modify the host response,[7,8] it is also clear that a specific microbial challenge is necessary for development of disease.[9] Gingivitis is considered as the stage that precedes periodontitis[10]; however, the manner by which the gingivitis-associated microbiota contributes to the initiation of periodontitis has not been elucidated. A better understanding of the intermicrobial interactions and the ecological events that promote the transitions from one dysbiotic stage to the next could lead to development of microbiome-targeted therapies to prevent periodontitis. In this manuscript we present a review of the compositional differences of subgingival communities associated with health, gingivitis, and periodontitis. As we found only 116S ribosomal RNA gene-based study, with a small sample size, that directly compared the 3 conditions,[11] we reanalyzed publicly available 16S ribosomal RNA gene datasets. We implemented sample-inclusion criteria that are compatible with current (at the time of writing) definitions of periodontal health, gingivitis, and periodontitis, and reprocessed and reclassified sequences under unified bioinformatic parameters, which allowed direct comparison of studies. This unified 16S ribosomal RNA gene dataset was interrogated to define the microbial signatures of health, gingivitis, and periodontitis. The most abundant community members under each state were defined, as well as the species enriched and those that do not change in proportion (core species) when one state is compared with another. We then evaluated the frequency of detection of periodontitis-associated taxa in health and gingivitis and conducted a network correlation analysis to

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explore species co-occurrence patterns under each state. We fully acknowledge the limitations of combining datasets produced under different experimental conditions. However, it was evident, through our analysis, that periodontal status was a greater determinant of microbiome composition and community structure than the study from which samples originated. Our reanalysis of publicly available datasets provides a useful framework to understand species shifts during the different stages of subgingival dysbiosis.

2 | RELEVANCE OF EXAMINING COMPOSITIONAL SHIFTS OF THE SUBGINGIVAL MICROBIOME

Development of gingivitis is a response to an increased load of subgingival microorganisms and to accompanying changes in the composition of the subgingval microbial community. Lack of adequate oral hygiene inevitably leads to specific and reproducible microbial successions conducive to an increase in the proportional counts of gram-negative, in some cases motile, microorganisms.[12-14] These changes in composition of the oral microbiota probably result from the formation of new niches by colonizing bacteria, which create environments propitious for the establishment of successive "waves" of species. Innate immune ligands abundant in gram-negative bacteria, such as lipopolysaccharide and serine-containing lipids, activate inflammatory responses and are likely to trigger gingivitis as a result of their increased load.[15-17] During gingivitis, the inflammatory exudate flowing through the gingival sulcus favors the growth of proteinase-rich gram-negative bacteria that utilize glycoproteins as their main source of energy.[18-20] Therefore, as outlined in the Ecological Plaque Hypothesis,[21] changes in the composition of dental biofilms induce inflammation, which in turn perpetuates these compositional changes. Longitudinal clinical studies show that gingivitis is a risk factor for periodontitis,[10,22] but whether, and in what manner, the gingivitis-associated microbiota promotes periodontitis is not known.

As in gingivitis, tissue destruction associated with periodontitis has been shown to correlate with specific shifts in the composition of subgingival microbiome communities. Evidence supporting the notion that specific microbial stimuli initiate periodontitis includes the results of human clinical longitudinal studies, which show that a dysbiotic microbiome community with enrichment of certain species, virulence factors, and/or metabolic activities precedes progression of periodontal disease.[10,23-27] Animal experiments, using a ligature-induced murine model of periodontitis and treatment with antibiotics that selectively deplete the microbiota, show that increased microbial load is essential for triggering disease-associated T-helper 17 immune responses which drive bone loss, but increased microbial load is insufficient to induce disease unless accompanied by specific alterations to the overall structure of the microbial community.[9] Other studies utilizing animal models also support the notion that specific species trigger disruptions in homeostasis, leading to tissue destruction. Mice orally inoculated with *Porphyromonas gingivalis* show evidence of bone loss, although *P gingivalis* only colonizes the murine oral cavity at low abundance.[28] In this model, *P gingivalis* is shown to dysregulate, in a protease-dependent manner, the complement cascade and block neutrophil phagocytosis, thereby promoting the overall growth of the microbial community, ultimately leading to inflammatory responses and bone loss.[28,29] In ecology, species that have an effect on the overall community which is disproportionally larger than the biomass they occupy, are called keystone species.[30] Therefore, *P gingivalis* has been called a "keystone pathogen", that is, "a keystone species which supports and shapes a microbial community in ways that also promote disease pathogenesis."[28] Although *P gingivalis* is indigenous to the oral cavity of humans, it is only found to colonize, at detectable levels, a small fraction of periodontally healthy subjects.[31,32] Colonization of *P gingivalis* and other keystone pathogens may be favored by the development of gingivitis. Once keystone pathogens colonize in low abundance, they may contribute to the initiation of human periodontitis by dysregulating host-protective responses. Ultimately, as tissue destruction occurs and periodontitis lesions establish, keystone pathogens may become abundant, numerically dominant, species, as seen in certain subjects affected by periodontitis who harbor a high proportion of *P gingivalis*.[33] However, as postulated in the Polymicrobial Synergy and Dysbiosis model,[34] the dysbiotic microbiome shifts detected in sites of periodontal breakdown are a consequence of interbacterial synergism and environmental perturbations caused by immune dysregulation and inflammation.[18,19,34,35] Therefore, although specific species may initiate or drive destructive tissue responses, the existence of those "driver" species depends on other community members, and the "driver" species may, in turn, influence the biomass of other bystander taxa. Thus, the dysbiotic community as a whole represents a challenge to the adjacent gingiva.

Gingivitis and periodontitis are considered to be successive stages of the same disease process; however, it is not clear to what extent the health- and gingivitis-associated microbiota are protective and exclude pathobionts, and whether the health- and gingivitis-associated microbiota precede, and are required to facilitate the growth of, periodontitis-associated taxa. To better understand the dynamics of the microbial stimuli that trigger disruptions in periodontal homeostasis, it seems essential to define the specific microbial signatures associated with the different stages of periodontal dysbiosis. Knowledge is needed on the interspecies interactions—synergistic or antagonistic—that occur among components of the microbiome at different stages of gingivitis and periodontitis. It would also be of interest to evaluate the presence and levels of periodontitis-associated species in gingivitis and health to understand whether gingivitis and periodontitis represent a continuum. Studies to evaluate if specific subgingival species or communities trigger, directly or indirectly, immune responses conducive to connective tissue attachment and bone loss could also benefit from a better definition of the different stages of subgingival dysbiosis. Identification of the microbial signatures of health, gingivitis, and periodontitis could also aid in the evaluation of microbiological outcomes after different periodontal therapies. The therapeutic modalities currently used do

not seem effective at completely resolving dysbiosis and reconstituting a health-compatible community.[36] However, the evaluation of therapeutic outcomes has been, for the most part, conducted using targeted microbiological techniques and not via whole-microbiome evaluation. The benefits of different therapeutic modalities at a microbiome community level are unclear, and it is uncertain whether the communities obtained after periodontal therapy resemble those found in health or in gingivitis, or still fall under the global characteristics of a periodontitis-associated community.

3 | MICROBIOME STUDIES COMPARING HEALTH, GINGIVITIS, AND PERIODONTITIS

We reviewed the current literature describing the microbial signatures associated with health, gingivitis, and periodontitis. Only 1 study—that by Park et al[11]—has directly compared these 3 states using molecular evaluation of the microbiome; by contrast, a variety of 16S ribosomal RNA gene-based studies have compared health with periodontitis or have characterized the shifts in microbiome occurring during natural or experimental gingivitis but failed to include direct comparison with the microbiome found in periodontitis.[33,37-43] The study by Park et al[11] included a small number of subjects (32 Korean individuals), in whom a distinct microbiome was observed according to periodontal health status. Samples from subjects with gingivitis had higher alpha (within-sample) diversity than those from periodontally healthy subjects or subjects with periodontitis. Healthy communities were dominated by *Halomonas hamiltonii*, which is not typically reported in the oral cavity but was thought to colonize this Korean population because of their eating habits. High levels of the genera *Porphyromonas* (32.2%), *Fretibacterium* (10.4%), *Rothia* (5.3%), and *Filifactor* (3.1%) were observed in periodontitis, while the genera *Streptococcus* (20.0%), *Capnocytophaga* (7.0%), *Haemophilus* (5.5%), and *Leptotrichia* (4.9%) dominated gingivitis communities.

Other studies in the literature have evaluated the subgingival communities in periodontal health,[44] compared the subgingival microbiome of a periodontally healthy state with that found in gingivitis,[37,38,43] or compared periodontally healthy individuals with those with periodontitis.[33,39-42,45-47] The characterization of healthy subjects in the Human Microbiome Project revealed subgingival communities rich in gram-positive species from the genera *Rothia*, *Actinomyces*, *Streptococcus*, and *Corynebacterium*, among others, with a few gram-negative taxa also present in high abundance, including *Fusobacterium nucleatum*, *Veillonella parvula*, and *Capnocytophaga* spp.[44] Studies comparing communities from periodontally healthy subjects with those from subjects with periodontitis show a variety of taxa associated with health and depleted in periodontitis, including *Actinomyces*, *Rothia*, and *Streptococcus* spp, among others (reviewed in Diaz et al[6]). Communities of higher diversity than those found in health are associated with periodontitis and enriched for a variety of species, including *P gingivalis*, *Porphyromonas endodontalis*, *Tannerella forsythia*, several *Treponema* spp, *Filifactor alocis*, *Prevotella intermedia*, *Parvimonas micra*, and *Fretibacterium* spp, among others (reviewed in Diaz et al[6]). Studies in which the microbiome signatures in gingivitis were evaluated include a study by Huang et al,[38] of 50 adults with gingivitis. In this study, baseline samples (ie, natural gingivitis state) were obtained from all participants. This was followed by an oral-hygiene treatment phase to restore gingival health, then by a 3-week period of no oral hygiene in order to induce experimental gingivitis. This investigation found similar communities in natural and experimental gingivitis, in agreement with previous cultivation studies.[5] At phylum level, Actinobacteria and Firmicutes were associated with a healthy gingiva, and TM7, Bacteroidetes, and Fusobacteria were enriched in gingivitis.[38] Twenty-two genera, namely *Leptotrichia*, *Prevotella*, *Fusobacterium*, TM7 (*Saccharibacteria*), *Porphyromonas*, *Tannerella*, *Selenomonas*, uncultured *Lachnospiraceae*, unclassified *Comamonadaceae*, *Peptococcus*, *Aggregatibacter*, *Catonella*, *Treponema*, SR1 (*Absconditabacteria*), *Campylobacter*, *Eubacterium*, *Peptostreptococcus*, unclassified *Bacteroidaceae*, *Solobacterium*, *Johnsonella*, *Oribacterium*, and unclassified *Veillonellaceae*, were associated with gingivitis.[38] Using an experimental gingivitis model similar to that of Huang et al,[38], our group characterized evolution of the microbiome in 15 subjects during 3 weeks of plaque accumulation.[37] We found that after 1 week of no oral hygiene, and before clinical inflammation was evident, there was a decrease in *Rothia* and an increase in *Veillonella*. After 3 weeks of no oral hygiene, we found enrichment of the genera *Prevotella*, *Mogibacterium*, *Fusobacterium*, and *Alloprevotella*, among others, while *Rothia* remained the taxon most strongly associated with health.[37] Although there is overlap among different studies in some of the genera associated with gingivitis and periodontitis, close examination of the species reported suggests that taxa enriched in gingivitis and periodontitis are, for the most part, different.[6]

4 | REANALYSIS OF PUBLICLY AVAILABLE DATASETS TO DEFINE THE MICROBIAL SIGNATURES OF HEALTH, GINGIVITIS, AND PERIODONTITIS

Our review of the literature shows a need to conduct a direct comparison of health, gingivitis, and periodontitis in a large number of individuals to better define the specific microbial signatures in each state. To start addressing this knowledge gap, we conducted an evaluation of the subgingival microbiome in health, gingivitis, and periodontitis by combination and reanalysis of publicly available 16S ribosomal RNA gene datasets. We fully acknowledge the limitations of combining sequence datasets generated by different investigators who used their own sampling and sequencing methods. Our combined reanalysis, however, represents a first step toward identifying the microbial signatures of health, gingivitis, and periodontitis. An advantage of reanalyzing these datasets together, rather than just comparing the species reported in the original manuscripts, is the use of unified processing and classification methods for sequence reads. Investigators use different reference databases for species classification, and therefore the same sequence could be reported

TABLE 1 Inclusion criteria, clinical parameters, and sample characteristics of studies included in the reanalysis of 16S ribosomal RNA gene-based sequencing datasets

	Study (Reference)	Inclusion criteria	Clinical parameters (mean ± SD)
Health	Abusleme et al (39)	• Nonsmokers • At least 14 teeth, excluding third molars, and ≥10 posterior teeth • No periodontal treatment before the time of the examination • No systemic illness • Not pregnant • No antibiotics, anticoagulants or use of NSAIDs within last 6 mo • ≥90% of sites with PD and CAL ≤3 mm • No site with PD >4 mm • BoP in <10% of sites	Full-mouth PD = 1.5 ± 0.1 mm Full-mouth CAL = 1.4 ± 0.2 mm Full-mouth % BoP = 4.4 ± 4.3% Full-mouth % of sites with visible plaque = 16.1 ± 13.4%
	The-Human-Microbiome-Consortium (44)	• 18-40 y old • No antibiotic use within the last 6 mo • No chronic medical conditions • No site with PD ≥4 mm • ≤8 missing teeth • BoP in ≤10% of sites	Not reported
Gingivitis	Schincaglia et al (37)	• ≥21 y old • No gingival or mucosal infection at sampled sites • No radiographic bone loss at sampled sites • No antibiotic, chronic anti-inflammatory medication, and anticoagulants within the last 6 mo • No medication initiated <3 mo prior to the screening visit • No medications known to affect periodontal health • No impaired kidney function, heart murmur, rheumatic fever, or bleeding disorder • No hepatitis, tuberculosis, or HIV • No caries or periodontal disease • No smoking • Not pregnant or planning to become pregnant in the following 3 mo • PD ≤4 mm at sampled sites	Not reported
	Huang et al (38)	• ≥18 y old • At least 12 natural anterior teeth • At least 5 sites showing bleeding as measured by MGI at the initial visit (Day-21 NG) • In good general health • No severe periodontal disease (defined as ≥4 teeth in 2 quadrants with pockets >5 mm, purulent exudates, generalized mobility, and/or severe recession) • Any condition that requires antibiotic premedication for administration of dental prophylaxis • No self-reported pregnancy or intent to become pregnant during the course of the study and no breast-feeding women • No fixed facial orthodontic appliances, atypical discoloration or pigmentation in the gingival tissue or any diseases or conditions that could be expected to interfere with the participant safely completing the study	NG: • Full-mouth % BoP = 13.5 ± 5.12% • MGI = 1.61 ± 0.24 EG: • Full-mouth % BoP = 26.00 ± 9.59% • MGI = 2.12 ± 0.48
Periodontitis	Abusleme et al (39)	• Nonsmokers • At least 14 teeth, excluding third molars, and ≥ 10 posterior teeth • No periodontal treatment before the time of the examination • No systemic illness • Not pregnant • No antibiotics, anticoagulants, or use of NSAIDs within the last 6 mo • ≥5 teeth with PD ≥ 5 mm and CAL ≥ 4 mm • BoP in at least 20% of sites • Radiographic evidence of bone loss	Full-mouth PD = 3.0 ± 0.7 mm Full-mouth CAL = 3.9 ± 0.9 mm Full-mouth % BoP = 44.5 ± 18.6% PD of sampled sites = 7.0 ± 1.5 mm CAL of sampled sites = 8.0 ± 2.1 mm Full-mouth % of sites with visible plaque = 82.4 ± 9.6%

Sampling method	Number of subjects	Number of sites sampled	Samples pooled for sequencing	Number of samples available for analysis	Number of samples selected	Number of samples included after processing and normalizing by subsampling at 3500 reads
Curette	10	2 sites per subject	No	17	17	9
Curette	131	1 site per subject	No	79	79	77
Curette	15	1 site per subject	No	15 (EG) after 21 days of interrupted oral hygiene	15 (EG)	15 (EG)
Curette	50	All teeth in 2 different quadrants	Yes	50 (NG) 50 (EG)	50 (NG) 50 (EG)	20 (NG) 42 (EG)
Curette	22	2 sites per subject (1 bleeding and 1 nonbleeding)	No	22 (bleeding) 22 (nonbleeding)	44	16

(Continues)

TABLE 1 (Continued)

Study (Reference)	Inclusion criteria	Clinical parameters (mean ± SD)
Camelo-Castillo et al (41)	• 30-65 y old • Good general health • No pregnancy or breastfeeding • No intake of systemic antimicrobials during the previous 6 mo • No intake of anti-inflammatory medication within the last 4 mo • No routine use of oral antiseptics • No presence of implants or orthodontic appliances • No previous periodontal treatment • At least 18 natural teeth • BOP-1 group (mean BoP score ≤ 50% in sampled sites) • BOP-2 group (mean BoP score > 50% in sampled sites) • Diagnosed with moderate-to-severe generalized chronic periodontitis according to established criteria (55)	BOP-1: • Full-mouth PD = 3.51 ± 0.81 mm • Full-mouth CAL = 4.62 ± 1.45 mm • Full-mouth % BoP = 39.75 ± 19.17% • Full-mouth BPL = 58.50 ± 26.68% • CAL in sampled sites = 6.62 ± 1.78 mm • % BoP in sampled sites = 33.79 ± 15.49% BOP-2: • Full-mouth PD = 3.42 ± 0.67 mm • Full-mouth CAL = 3.88 ± 0.99 mm • Full-mouth % BoP = 57.32 ± 18.02% • Full-mouth BPL = 50.44 ± 24.09% • CAL in sample sites = 6.01 ± 0.94 mm • % BoP in sample sites = 79.78 ± 12.96%
Ganesan et al (46)	• No conditions that required the use of prophylactic antibiotics • No current or planned pregnancy • No HIV infection • No long-term (more than 3 mo) use of medications known to cause gingival changes, (eg, immunosuppressant, phenytoin, calcium channel blockers, aspirin, NSAIDs, bisphosphonates. or steroids) • No antibiotic therapy or oral prophylactic procedures within the last 3 mo • At least 20 teeth in the dentition • Attachment loss ≥ 5 mm, probing pocket depths ≥ 5 mm and mean gingival index >1 in 30% of more of sites	NSND: • Full-mouth PD = 6.0 ± 1.5 mm (deep sites), 1.99 ± 0.35 mm (shallow sites) • Full-mouth CAL = 6.7 ± 2.9 mm (deep sites), 1.1 ± 0.9 mm (shallow sites) • Full-mouth % BoP = 100% (deep sites), 6.0 ± 3.0% (shallow sites) NSD • Full-mouth PD = 5.62 ± 0.16 mm (deep sites), 2.17 ± 0.25 mm (shallow sites) • Full-mouth CAL = 4.75 ± 0.34 mm (deep sites), 1.75 ± 0.27 mm (shallow sites) • Full-mouth % BoP = 100% (deep sites), 6.0 ± 2.0% (shallow sites) SND • Full-mouth PD = 5.76 ± 0.17 mm (deep sites), 2.4 ± 0.29 mm (shallow sites) • Full-mouth CAL = 4.75 ± 0.5 mm (deep sites), 1.02 ± 0.3 mm (shallow sites) • Full-mouth % BoP = 100% (deep sites), 4.0 ± 3.0% (shallow sites) • SD Full-mouth PD = 5.82 ± 0.46 mm (deep sites), 1.9 ± 0.35 mm (shallow sites) • Full-mouth CAL = 5.32 ± 0.44 mm (deep sites), 0.73 ± 0.3 (shallow sites) • Full-mouth % BoP = 100% (deep sites), 5.0 ± 2.0% (shallow sites)

Sampling method	Number of subjects	Number of sites sampled	Samples pooled for sequencing	Number of samples available for analysis	Number of samples selected	Number of samples included after processing and normalizing by subsampling at 3500 reads
Paper points	60	8 nonadjacent proximal sites	Yes (per category and subject)	24 (BOP-1), 6 nonsmoker and 18 smokers 36 (BOP-2), 22 nonsmokers and 14 smokers	28 (nonsmokers)	2
Paper points	25 NSND 25 NSD 25 SND 25 SD	4 shallow sites and 4 deep sites per subject	Yes (per category and subject)	25 (NSND_D) 25 (NSND_S) 25 (NSD_D) 25 (NSD_S) 25 (SD_D) 25 (SD_S) 26 (SND_D)	25 (NSND_D)	25 (NSND_D)

(Continues)

TABLE 1 (Continued)

Study (Reference)	Inclusion criteria	Clinical parameters (mean ± SD)
Griffen et al (40)	• At least 35 y old • At least 20 teeth • No antibiotic therapy or professional cleaning within the last 3 mo • Not on immunosuppressant medications or steroids • No diabetes or HIV • At least 4 mm attachment loss and 5 mm probing depth in atleast 3 nonadjacent interproximal sites in at least 2 quadrants	PD sampled sites = 6.15 ± 1.30 mm (deep sites), 3.20 ± 0.52 mm (shallow sites) % BoP sampled sites = 52.9 ± 46% (deep sites), 29.9 ± 36.0% (shallow sites) CAL ≥ 4
Hong et al (33)	• No history of smoking • No antibiotic use within the last month • No periodontal treatment within 1 y • A minimum of 15 teeth • At least 1 site with PD of 5 mm • At least 2 interproximal sites with CAL of 6 mm	Full-mouth PD = 3.2 ± 0.8 mm Full-mouth CAL = 3.6 ± 1.0 mm Full-mouth % BoP = 44.0 ± 25.0% % of sites with PS = 66.0 ± 23.00
Kirst et al (42)	• No history of systemic diseases that could interfere with clinical characteristics, incidence, or progression of periodontal disease • No chronic treatment with any medication known to affect periodontal status within the previous 3 mo (ie, antibiotics, NSAIDs, and oral contraceptives) • Clinical diagnosis and selection of subjects were based on clinical and radiographic criteria proposed by the American Academy of Periodontology (55).	Not reported
Kistler et al (43)	• No systemic disease • No history of antibiotic use for at least 3 mo prior to the study • Not pregnant • No smokers • At least 20 teeth • At least 6 teeth with PD ≥6 m and bone loss	Not reported

Abbreviations: BPL, bacterial plaque level; BoP, bleeding on probing; CAL, clinical attachment loss; CKD, chronic kidney disease; EG, experimental gingivitis; MGI, Mazza Gingival Index; NG, natural gingivitis; NSD, nonsmoking, diabetic; NSD_D, nonsmoking, diabetic deep site; NSD_S, nonsmoking, diabetic shallow site; NSND, nonsmoking, nondiabetic; NSND_D, nonsmoking, nondiabetic deep site; NSND_S, nonsmoking, nondiabetic shallow site; PD, probing depth; PS, plaque score; SD, smoking, diabetic; SD_D, smoking, diabetic deep site; SD_S, smoking, diabetic shallow site; SND, smoking nondiabetic; SND_D, smoking nondiabetic deep site; SND_S, smoking nondiabetic shallow site.

under different names across studies. Moreover, even if studies used the same reference database for taxonomic assignments, such as the broadly used Human Oral Microbiome Database,[48] different versions of the database exist and are used across studies as oral microbial taxonomy is in constant evolution.

Our strategy to select the datasets included in this reanalysis was as follows:

1. Identification of studies that used 16S ribosomal RNA gene amplicon sequencing of the V1-V3 hypervariable regions to characterize the subgingival microbiome in health, gingivitis, or periodontitis. We included only studies that sequenced the most commonly used V1-V3 region. We acknowledge, however, that different primers were used for this region, possibly leading to different representation of selected taxa.[49-51] Through this step we identified 6 studies that included a cohort described as subgingival health,[11,39,40,42,44,46] 3 studies that included a cohort with either natural gingivitis or experimental gingivitis induced by 3 weeks of abstaining from oral hygiene,[11,37,38] and 8 studies that included a cohort with periodontitis.[11,33,39-43,46]
2. Identification of studies with downloadable, publicly available, sequence datasets. We identified, from the studies referenced above, those with publicly available sequence datasets. If clarification was required or if mapping information was missing from the public archive, we contacted the authors of each study directly. At this step, the following datasets were available: 4 for health,[39,40,42,44] 2 for gingivitis,[37,38] and 7 for periodontitis.[33,39-43,46]
3. From the studies identified above, we then selected cohorts for which a strict definition of health, gingivitis, or periodontitis was used for inclusion of subjects in each group. Periodontal health is defined as "a state free from inflammatory periodontal disease", which implies absence or only a minimal amount of inflammation in a periodontium with normal support.[52] Gingivitis is defined at a site level as gingival redness and edema in the absence of progressive periodontal attachment loss.[53] At a subject level, gingivitis is defined by the presence of bleeding on probing in ≥10% of sites.[53] Periodontitis is defined as "a microbially-associated, host-mediated inflammation that results in loss of periodontal attachment."[54] A recently proposed case definition of periodontitis includes interdental clinical attachment loss detectable at 2

Sampling method	Number of subjects	Number of sites sampled	Samples pooled for sequencing	Number of samples available for analysis	Number of samples selected	Number of samples included after processing and normalizing by subsampling at 3500 reads
Paper points	29	3 deep sites and 3 shallow sites per subject	Yes (per category and subject)	29 (deep sites) 29 (shallow sites)	29 (deep sites)	24 (deep sites)
Curette	34	The 2 sites with the deepest PDs in each subject	Yes (per subject)	34 in total: 9 subjects with CDK 3 with diabetes 8 with CDK and diabetes 14 with no CDK or diabetes	14	14
Paper points	25	2 sites per subject	Yes	50	50	16
Curette	20	Not reported	Yes	34	34	13

or more nonadjacent teeth, or buccal clinical attachment loss of ≥3 mm with pocketing of >3 mm detectable at 2 or more teeth.[54] We therefore selected cohorts that complied with these definitions. As seen in Table 1, only 2 studies included cohorts of subjects that could be classified as periodontally healthy and free of gingivitis (ie, no periodontitis and <10% of sites with bleeding on probing). It is also evident, in Table 1, that the definition of gingivitis used by Huang et al[38] may have included cases of initial periodontitis, as the selected subjects could have presented sites with probing depths equal to 5 mm. However, as only 2 cohorts were available in which gingivitis samples were included, we did not exclude this study. Table 1 also shows 7 studies, which were included, that reported on cohorts with periodontitis.

4. Within the studies chosen, we further selected samples to include only those from subjects who were nonsmokers, nondiabetic, and did not have chronic kidney disease. Therefore, the final samples selected for analysis only included nonsmoker, systemically healthy individuals. If any periodontitis studies included shallow and deep periodontitis sites, we only included the samples from deep sites in the analysis.

Downloaded sequences were processed in mothur.[56] The steps to process these datasets included a screening step to identify and retain reads without ambiguities, with a maximum of 8 homopolymers, and a length between 200 and 550 bp. Chimeric sequences were removed using UCHIME.[57] For the studies of Griffen et al[40] and Ganesan et al,[46] which included combined sequences of different hypervariable regions of the 16S ribosomal RNA gene, we performed additional processing steps to select only V1-V3 sequences. To exclude non–V1-V3 sequences, unique reads were aligned to the full-length 16S ribosomal RNA SILVA database (release 132),[58] followed by filtering out all sequences that did not align at the location of the V1-V3 region. Sequences were classified to species level using the classify.seqs command and the Human Oral Microbiome Database V14.5[59] as reference. Parameters used were method = knn, search = blast, gapopen = −5, gapextend = −5, match = 4, mismatch = −5, and numwanted = 1, following the recommendations of Al-Hebshi et al.[60] We validated this taxonomy assignment algorithm by classifying trimmed Human Oral Microbiome Database reference sequences against the Human Oral Microbiome Database full-length reference sequence database. With a few exceptions, all short

sequences extending over the V1-V2 region can be correctly classified. However, the following species cannot be discriminated from each other: *Lactobacillus casei* and *Lactobacillus rhamnosus*; *V parvula* and *Veillonella dispar*; *Streptococcus mitis*, *Streptococcus pneumoniae*, and *Streptococcus* sp HOT423; and *Neisseria flavescens* and *Neisseria subflava*. Counts for species that cannot be correctly identified were aggregated in the current analysis.

After processing, sequence libraries were randomly subsampled in order to ensure that they contain the same number of reads, and all analyses were conducted using normalized libraries. We chose a subsampling threshold of 3500 reads, as we have previously shown that a sequencing effort between 3000 and 5000 reads is sufficient to stabilize diversity estimators in oral microbiome samples.[61] We also performed rarefaction analyses, which revealed that most curves were starting to become asymptotic at 3500 reads, and therefore this was the threshold chosen for subsampling. The final number of samples included per study and per category after excluding samples with low numbers of reads is shown in Table 1. Table 2 provides information on the primer pairs used in each study, the 16S ribosomal RNA gene regions analyzed, and median sequence length after our reprocessing.

5 | DISTINCT RICHNESS AND COMMUNITY STRUCTURE IN HEALTH, GINGIVITIS, AND PERIODONTITIS

Figure 1 shows a comparison of community alpha-diversity in health, gingivitis, and periodontitis. As seen in Figure 1A, the number of species observed was lowest in health, higher in periodontitis, and highest in gingivitis. Community alpha-diversity, however, which is a measure of the number of species (richness) and their distribution (evenness), did not differ between health and periodontitis but was higher in gingivitis (Figure 1B,C). These findings are in agreement with those reported by Park et al,[11] who also observed a higher number of species and greater species diversity in gingivitis than in health and periodontitis. It should be noted that these similar findings are independent as the dataset generated by Park et al. was not included in our reanalysis. With respect to the comparison between health and periodontitis, the finding of higher richness in periodontitis agrees with the results from some of the studies included in the reanalysis.[39-41] A higher number of species detected in disease is consistent with the concept that subgingival dysbiosis occurs as a result of microbial successions without replacement of primary

TABLE 2 Sample processing information

Study (Reference)	DNA-extraction method	Primers and 16S ribosomal RNA gene region sequenced	Platform used for sequencing	Median read length after processing (bp)
Abusleme et al (39)	Modified DNeasy Blood and Tissue Kit (Qiagen) with lysozyme and proteinase K overnight incubation added	8F - 361R (V1-V2)	454-pyrosequencing	344
Camelo-Castillo et al (41)	MasterPure Complete DNA and RNA Purification Kit (Epicentre Biotechnologies) with lysozyme treatment added	8F - 533R (V1-V3)	454-pyrosequencing	473
Ganesan et al (46)	Qiagen DNA MiniAmp kit (Qiagen)	8F - 536R (V1-V3) 1099F - 1541R (V7-V9)[a]	454-pyrosequencing	440
Griffen et al (40)	Bead beating plus Qiagen DNA MiniAmp kit (Qiagen)	27F - 342R (V1-V2) Primers for V4[a]	454-pyrosequencing	227
Hong et al (33)	Modified DNeasy Blood and Tissue Kit (Qiagen) with lysozyme and proteinase K overnight incubation added	8F - 361R (V1-V2)	454-pyrosequencing	258
Huang et al (38)	Modified DNeasy Blood and Tissue Kit (Qiagen) with bead beating, lytic enzyme cocktail (Lysozyme, mutanolysin, Lysostaphin) and extraction using Qiacube	5F - 534R (V1-V3)	454-pyrosequencing	322
The-Human-Microbiome-Project (44)	MO BIO PowerSoil DNA Isolation Kit (MO BIO Laboratories)	27F - 534R (V1-V3)	454-pyrosequencing	274
Kirst et al (42)	MO BIO Power Soil DNA extraction kit (MO BIO Laboratories)	27F - 534R (V1-V3)	454-pyrosequencing	520
Kistler et al (43)	GenElute Bacterial DNA Extraction Kit with lysozyme treatment added	27F - 534R (V1-V3)	454-pyrosequencing	515
Schincaglia et al (37)	Modified DNeasy Blood and Tissue Kit (Qiagen) with lysozyme and proteinase.K overnight incubation added	8F - 361R (V1-V2)	Illumina MiSeq	316

[a]Only reads overlapping the V1-V3 region were included in the analysis.

colonizers.[39] However, while our finding, of similar diversity in health and periodontitis, agrees with some studies,[42] it disagrees with other, previous, reports.[39,40] Overall, it appears that there is an increase in both richness and diversity of the subgingival microbiome during gingivitis, and while richness remains high in periodontitis because no species are lost during the shift, it seems that some species become dominant (ie, increase in proportion) in periodontitis-associated communities, decreasing community evenness and therefore reducing the overall diversity compared with gingivitis.

Next, we evaluated differences in global community structure. We compared distances among samples using the $Theta_{YC}$ index, which provides a measure of the difference between 2 communities based on the species present and their distributions. Using the $Theta_{YC}$ index, we evaluated whether samples clustered according to study or periodontal health status. Figure 2A-C shows that the study from which samples originated from was a determinant of community structure. However, as seen in Figure 2D, there was distinct separation of communities according to periodontal health status, with health, gingivitis, and periodontitis communities forming clearly distinct data clouds. These findings again confirm those of Park et al,[11] who directly compared communities in health, gingivitis, and periodontitis and showed that the community structure differs in the 3 states.

6 | SPECIES ASSOCIATED WITH HEALTH

Next, we determined which species characterized each state by comparing their relative abundances using the LEfSe tool.[62] We used stringent thresholds to select only the species with the most significant differences and the greatest effect size. The thresholds used were 3.5 for the logarithmic linear discriminant analysis score of discriminative features and 0.01 for the alpha value of the factorial Kruskal-Wallis test. Figure 3 shows the species enriched in health versus gingivitis, while Figure 4A depicts the most abundant species in health. A common metabolic feature of some of the taxa enriched in health is their high tolerance for oxygenated environments. *Rothia* spp, *Corynebacterium* spp, *Lautropia mirabilis*, *Neisseria flava*, *Bergeyella* spp, and *Kingella oralis* are considered as aerobes, while *Streptococcus*, *Actinomyces*, *Gemella*, and *Capnocytophaga* are considered to be facultative anaerobes. This is consistent with the high oxygen levels to which health-associated communities are exposed.[63,64] Taxa such as *Rothia* have also been shown to participate in cell-cell coaggregation interactions in early biofilms,[65] while *Corynebacterium* have been shown to serve as a key forming element in a spatially organized microbial consortium observed to occur during early biofilm formation.[66] Therefore, the species associated with health appear to be species with demonstrated roles during early biofilm colonization. This is consistent with the idea that a healthy state is maintained by frequent removal of oral biofilms via oral hygiene measures.

7 | SPECIES ASSOCIATED WITH GINGIVITIS

In contrast to health, communities in gingivitis are enriched mostly for gram-negative anaerobic species, although oxygen consumers, such as *Neisseria* spp and *Streptococcus* spp, are also part of the taxa enriched (Figure 3). This is consistent with the formation of a new niche in gingivitis that supports the growth of oxygen-sensitive anaerobes. Interestingly, 11 species of *Leptotrichia* appeared to be enriched in gingivitis (Figure 3), in agreement with previous reports.[11,38] *Leptotrichia* also appear to be some of the most abundant community members (Figure 4B). As gingivitis is associated with an increase in the total microbial load, this implies a change in the total

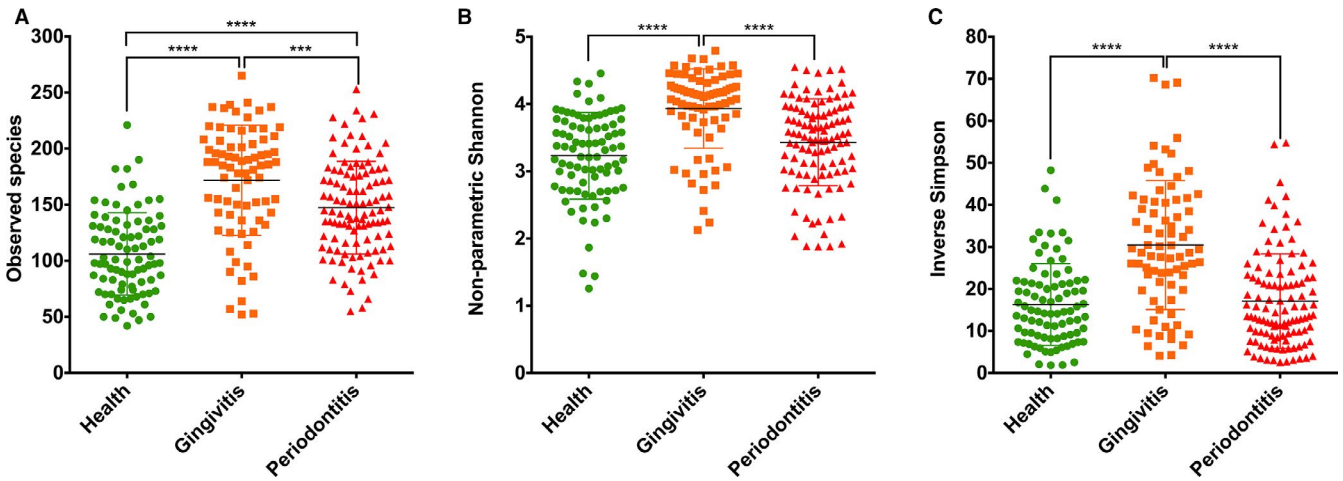

FIGURE 1 Alpha diversity estimates for microbial communities from health, gingivitis, and periodontitis. A, Number of bacterial species observed. Data were analyzed using one-way analysis of variance and Tukey's multiple comparisons test: ***$P < .001$ and ****$P < .0001$. B, Nonparametric Shannon diversity index. Data were analyzed using the Kruskal-Wallis test and Dunn's multiple comparisons test: ****$P < .0001$. C, Inverse Simpson diversity index. Data were analyzed using the Kruskal-Wallis test and Dunn's multiple comparisons test: ****$P < .0001$

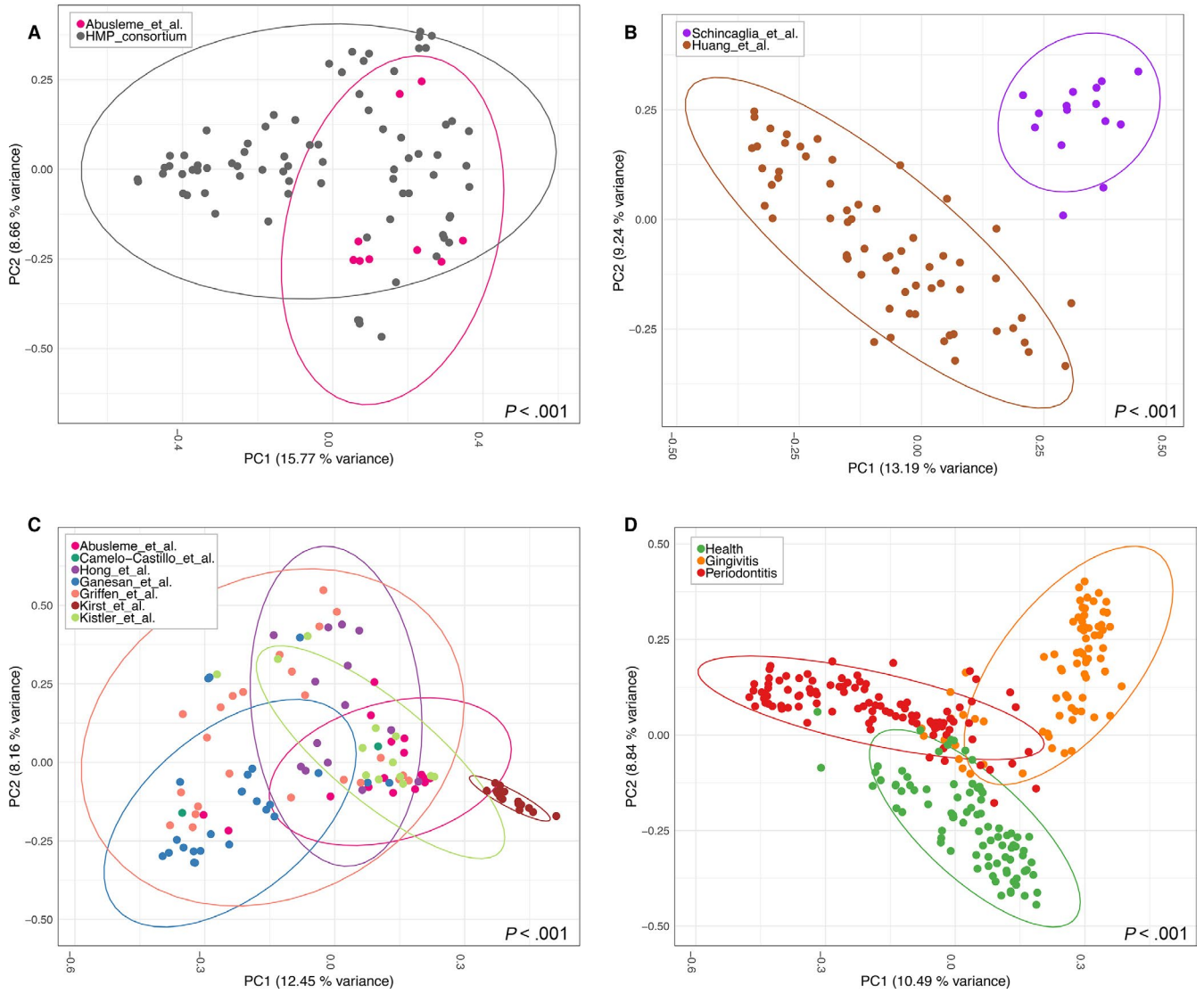

FIGURE 2 Bacterial community structure in health, gingivitis, and periodontitis. Principal coordinates analyses (PCoA) plots were constructed based on the $Theta_{YC}$ distance (a measure of community structure). PCoA plots show clustering of microbial communities within health (A), gingivitis (B), or periodontitis (C), according to the study from which samples were obtained. (D) The PCoA graph depicts significant separation of microbial communities according to periodontal condition, with samples clearly segregating in 3 distinct clusters (health, gingivitis, and periodontitis). Data clouds are shown with 95% confidence ellipses. Significance (*P*) of separation of data clouds was analyzed using analysis of molecular variance

burden of these gram-negative species that is even greater than that due to their proportional increase.

Of interest are the shifts in abundance of different species of *Fusobacterium* and subspecies of *F nucleatum* from health to gingivitis. *Fusobacterium nucleatum* subsp *vincentii* is highly abundant and enriched in healthy communities, but *Fusobacterium nucleatum* subsp *polymorphum* and *Fusobacterium periodonticum* become enriched in gingivitis (Figure 3). Besides the gingivitis studies included in this reanalysis,[37,38] *F nucleatum* subsp *polymorphum* was reported as enriched in gingivitis in the 2-week experimental study conducted by Kistler et al.[43] Although *F nucleatum* subsp *polymorphum* remains enriched in periodontitis compared with health, its abundance is lower than in gingivitis. Other *F nucleatum* subspecies become enriched in periodontitis (Figures 5 and 6). The specific virulence attributes that allow *F nucleatum* subsp *polymorphum*, but no other subspecies of *F nucleatum*, to become enriched during gingivitis are not known.

8 | SPECIES ASSOCIATED WITH PERIODONTITIS

During periodontitis, bacterial communities exhibit a profound shift characterized by enrichment of mostly gram-negative anaerobic taxa. This shift is distinct to that from health to gingivitis and, for the most part, the species enriched in periodontitis in comparisons with health or with gingivitis are congruent (Figures 5 and 6). Several species that have been classically associated with periodontitis are consistently overrepresented in both comparisons, such as *P gingivalis*,

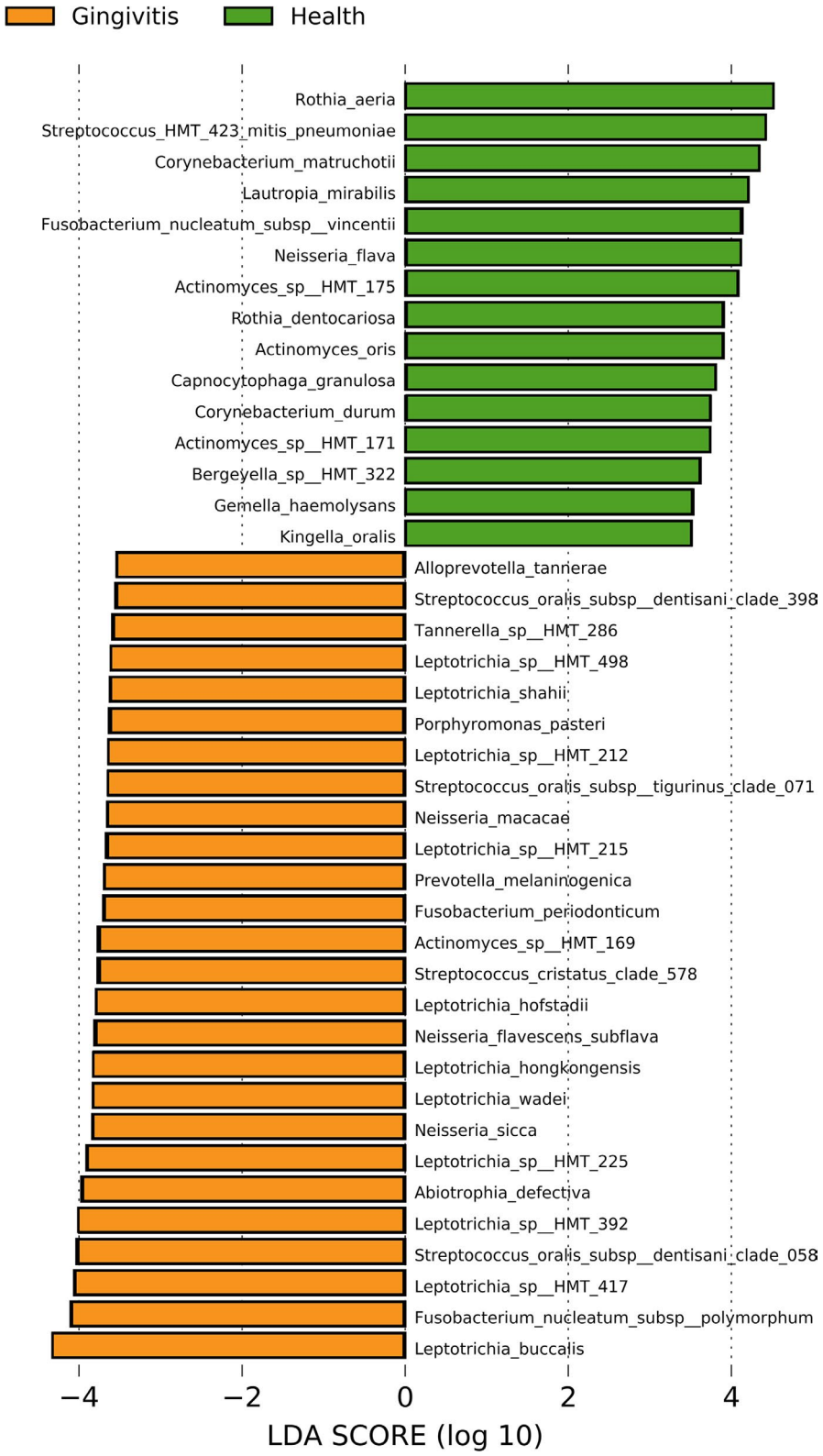

FIGURE 3 Bacterial species enriched in health or gingivitis. Samples from subjects with health and gingivitis were compared using LEfSe; the bars depict bacterial species with significantly different relative abundance in health and gingivitis. Bars represent linear discriminant analysis (LDA) scores

F alocis, *T forsythia*, *Treponema denticola*, and *P micra*, among others. Other uncultivable taxa enriched in periodontitis compared with health and gingivitis include *Treponema* sp HMT 237, *Fretibacterium* sp HMT 360, *Fretibacterium* sp HMT 361, and *Saccharibacteria* (TM7) [G1] HMT 349. In addition, most of these enriched species were also found in high abundance in periodontitis communities (Figure 4C). These results show that during periodontitis, the microbial burden associated with established lesions is dramatically distinct from that associated with gingivitis.

We also observed increased abundance of *Fusobacterium* spp during periodontitis relative to the abundance in health and gingivitis (Figures 4C, 5 and 6). Certain *Fusobacterium* spp were previously

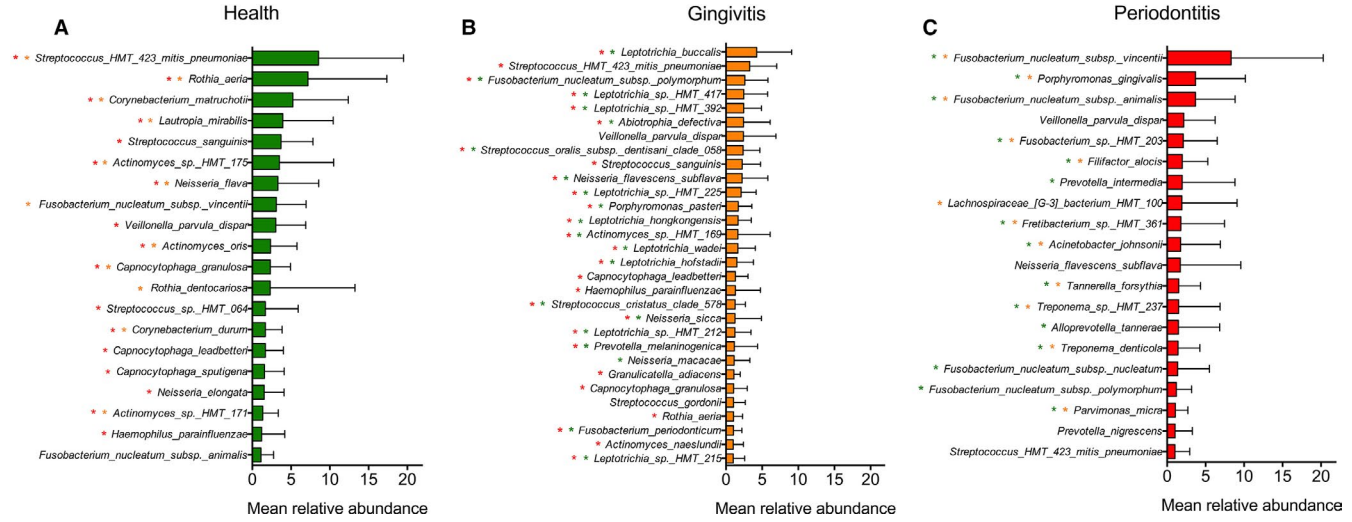

FIGURE 4 Community members most abundant in health, gingivitis, and periodontitis. The bar charts show bacterial species with relative abundance of more than 1% in health (A), gingivitis (B), and periodontitis (C). Bar represents mean and line is standard deviation. *The species was enriched in that condition when compared, using LEfSe, with the other two conditions (per analyses shown in Figures 3, 5 and 6). A green asterisk indicates that the species was enriched compared with health; an orange asterisk indicates that the species was enriched compared with gingivitis; and a red asterisk indicates that the species was enriched compared with periodontitis

identified as core species (ie, species whose proportion does not change from health to disease).[33,39] In the present analyses, all *F nucleatum* subspecies were enriched in periodontitis (Figure 7). *Fusobacterium nucleatum* subsp *vincentii* and *Fusobacterium nucleatum* subsp *animalis*, however, are still abundant components of healthy communities (Figure 4A). Moreover, with the exception of *F nucleatum* subsp *polymorphum*, the three other *F nucleatum* subspecies were detected with equal frequency in health, gingivitis, and periodontitis (Figure 7). Therefore, these results still support the concept that *F nucleatum* is an important component of subgingival dental plaque, possibly serving as a metabolic anchor for other community members.

9 | PREVALENCE OF PERIODONTITIS-ASSOCIATED SPECIES IN HEALTH AND GINGIVITIS

Next, we interrogated the frequency of detection of periodontitis-associated species in health and gingivitis. All periodontitis-associated species could be detected in gingivitis and most were also detected in health (Figure 7). Their frequency of detection in health was much lower, however, than their frequency of detection in gingivitis. Periodontitis-associated species with the lowest frequency of detection in health were *Fretibacterium* spp HMT 361 (not detected in health) and *P gingivalis* (only detected in 1 subject). Notably, both species become some of the most frequently detected and abundant members of periodontitis communities (Figure 4C). It is worth noting that a subset of species which were enriched and frequently detected in periodontitis were also highly prevalent in both health and gingivitis. Among these taxa were *Campylobacter gracilis* and various *F nucleatum* subspecies (*vincentii*, *animalis*, and *nucleatum*). Overall, the results from Figure 7 support the notion that periodontitis-associated species are indigenous and part of health-associated communities, becoming enriched as the environment changes during inflammation.[67] Gingivitis and periodontitis appear as a continuum, with gingivitis communities supporting increased levels (and therefore increased detection) of those species that ultimately dominate the dysbiotic communities associated with periodontitis. There appear to be differences, however, in the level of change of species from health to the inflammatory states with *Fretibacterium* sp HMT 361 and *P gingivalis* showing the most drastic increase in detection levels during inflammation.

10 | INTEGRATED OVERVIEW OF SUBGINGIVAL MICROBIOME SHIFTS IN RELATION TO HEALTH, GINGIVITIS, AND PERIODONTITIS

Figure 8 presents a summary of the results from this reanalysis, showing the species enriched (with increased relative abundance) in one state compared with the other two. Species in the overlapping area between 2 circles are those that were enriched in both comparisons and therefore could be considered as the signature species for each periodontal condition. It is evident from this figure that unique species are associated with each periodontal state and that although both gingivitis and periodontitis represent inflammatory states, their microbial signatures are distinct.

Species in the gray circles are those that do not change between groups (in pairwise comparisons) and that were also present in at least 70% of subjects included in the comparison (core species). Of these, *V parvula*, *V dispar*, *C gracilis*, and *Prevotella oris* were present at a similar

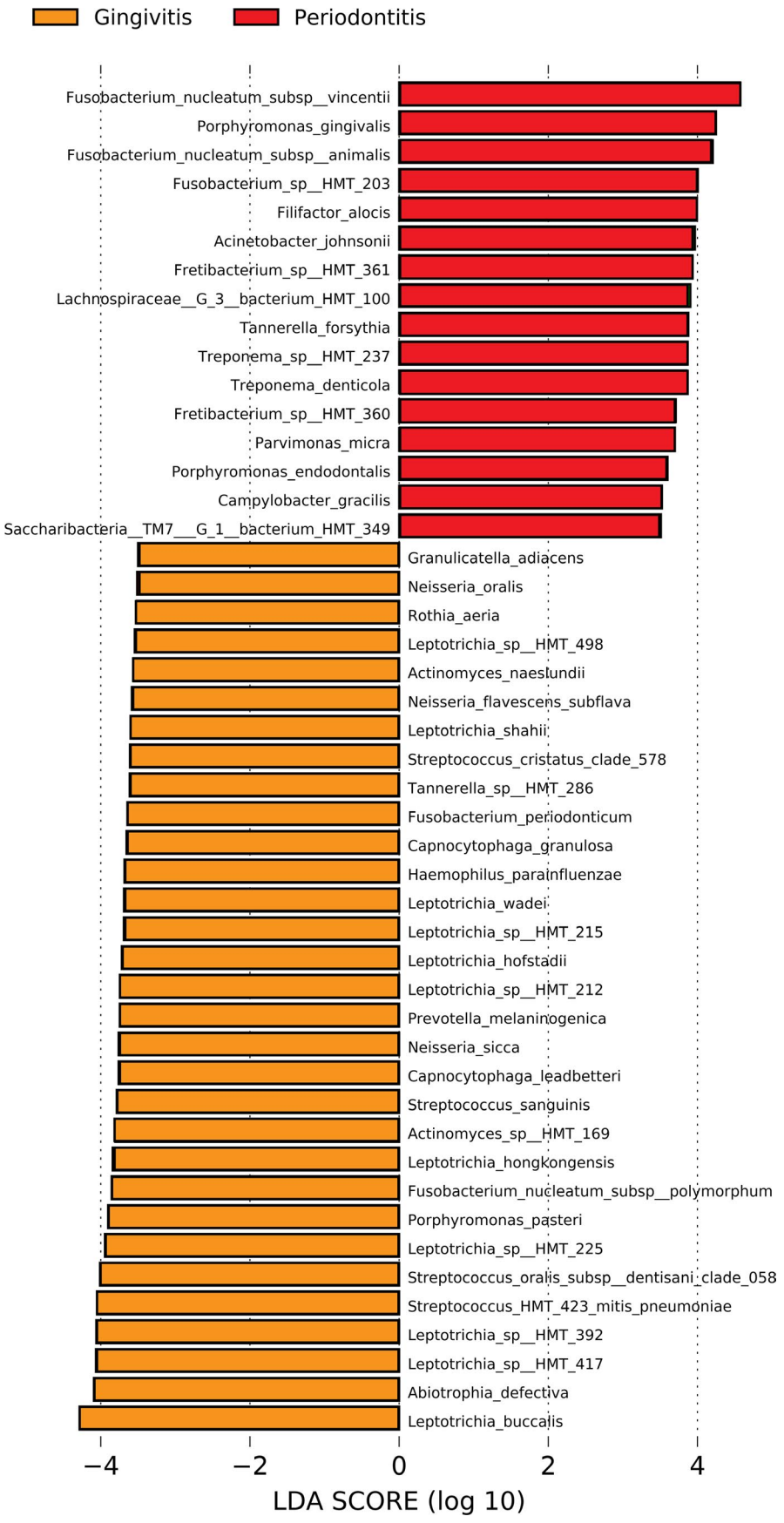

FIGURE 5 Bacterial species enriched in gingivitis or periodontitis. Samples from sites with gingivitis or periodontitis were compared using LEfSe; the bars depict bacterial species with significantly different relative abundance. Bars represent linear discriminant analysis (LDA) scores

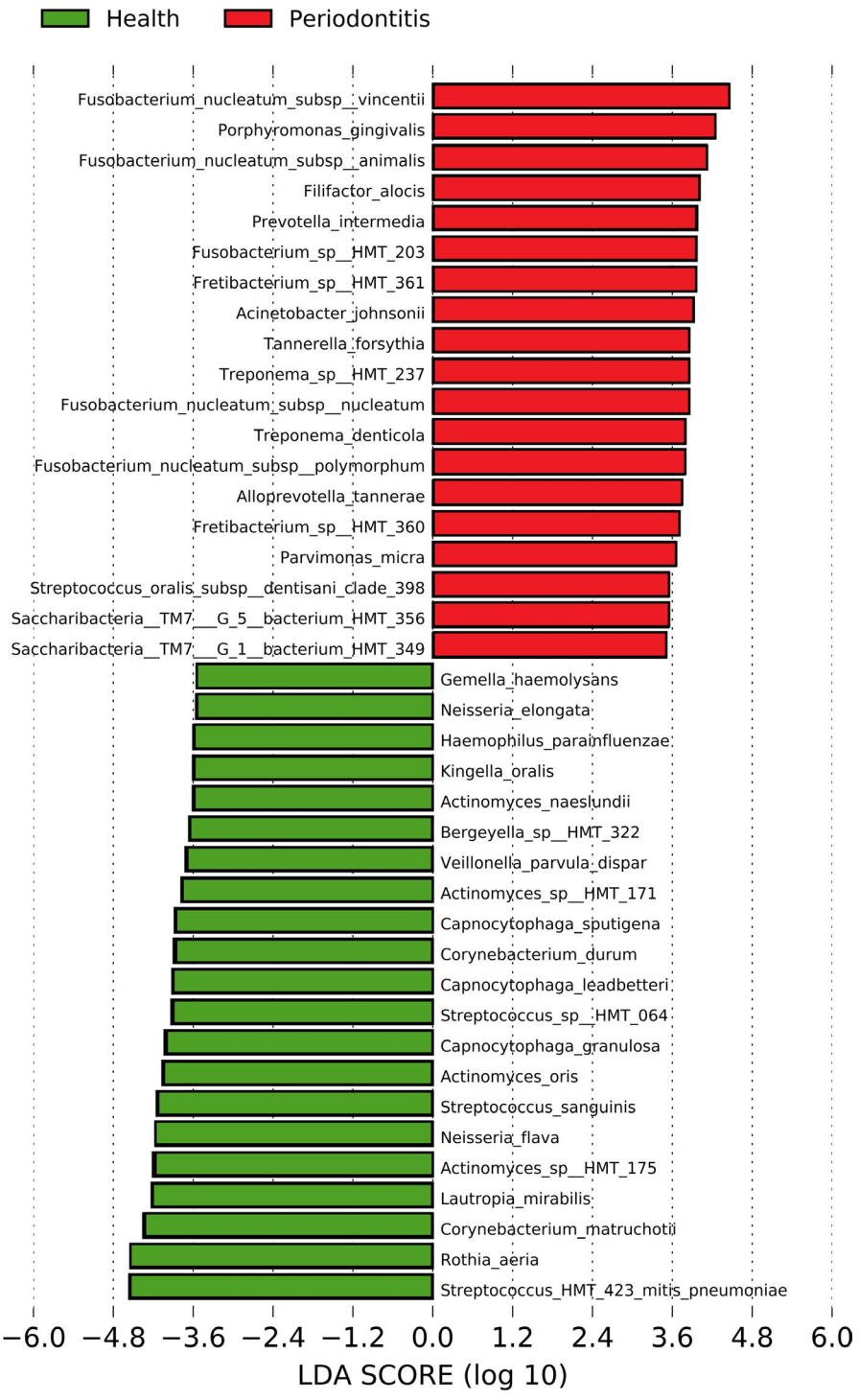

FIGURE 6 Bacterial species enriched in health or periodontitis. Samples from healthy periodontium or sites with periodontitis were compared using LEfSe; the bars depict bacterial species with significantly different relative abundance. Bars represent linear discriminant analysis (LDA) scores

relative abundance when comparing health with gingivitis and gingivitis with periodontitis. *Veillonella parvula*, *V dispar*, and *C gracilis* have been previously reported as belonging to this core group,[6] and therefore these results confirm previous findings. Several core species that do not differ in proportion between health and gingivitis have been shown to be important mediators of plaque formation, aiding in the establishment of late-colonizing periodontitis-associated species. For instance, *Streptococcus gordonii* has been shown to facilitate the accretion in biofilms of the periodontitis-associated species *P gingivalis* in an exchange mediated by streptococcal 4-aminobenzoate/para-amino benzoic acid.[68] *Veillonella* spp, which utilize lactic acid and are capable of heme biosynthesis, are thought to be important in the establishment of multispecies communities containing periodontitis-associated taxa supporting them via the mentioned metabolic activities.[69,70] These studies support the concept that core species, which increase in biomass as subgingival microbial communities transition from one state to another, but do not change in proportion, act as facilitators of dysbiosis.

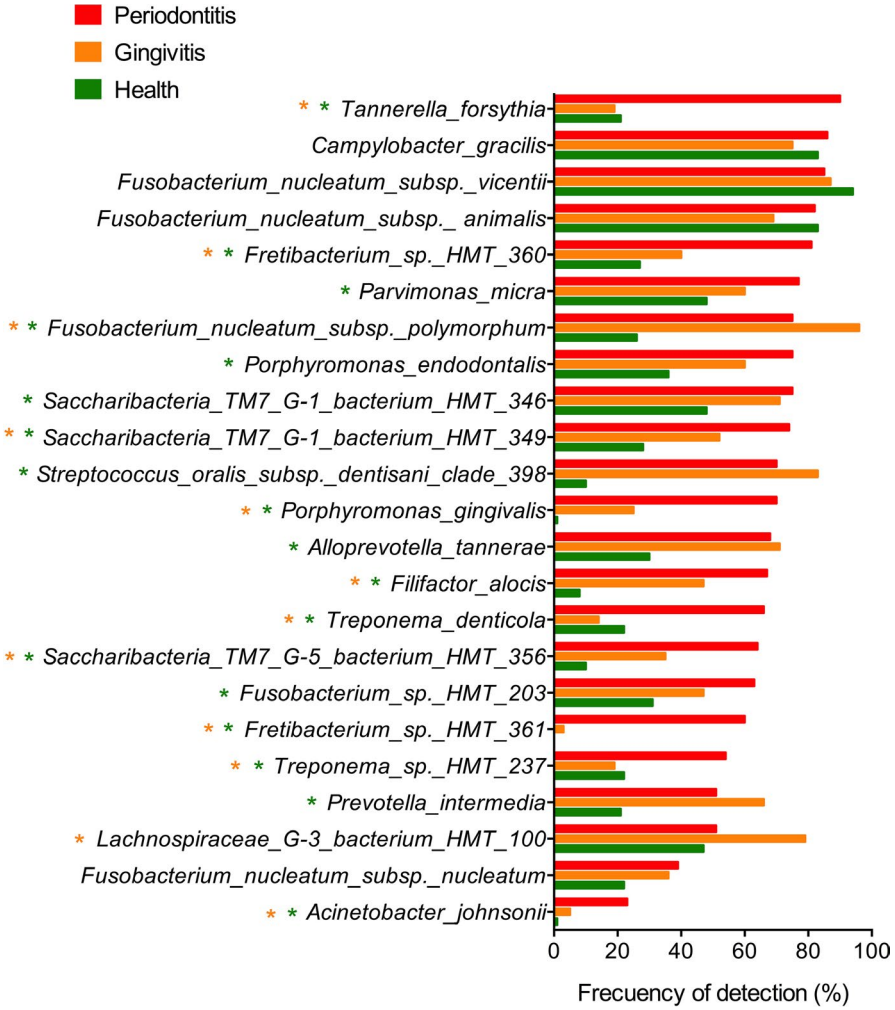

FIGURE 7 Frequency of detection of periodontitis-associated bacterial species in health and gingivitis. The bar chart shows bacterial species overrepresented in terms of relative abundance in periodontitis (determined using LEfSe). Bars represent percentage of samples in which the species was detected. *Species showing a different frequency of detection compared with health or gingivitis. Data were analyzed using the chi-square test. A green asterisk indicates species more frequently detected in periodontitis than in health, and an orange asterisk indicates species was more frequently detected in periodontitis than gingivitis

11 | NETWORK CO-OCCURRENCE ANALYSES OF COMMUNITIES ASSOCIATED WITH HEALTH, GINGIVITIS, AND PERIODONTITIS

We also conducted a network co-occurrence analysis to evaluate which species within each type of community (health, gingivitis, or periodontitis) showed positive or negative correlations. To avoid reporting spurious correlations, only species present in at least 50% of samples from each category were included in the analysis.[71] Abundance data were first transformed using a centered log-ratio procedure. R (http://www.R-project.org/) and Gephi[72] were used for correlation analysis (Spearman rank) and network visualization. Only strong correlations (with correlation coefficients larger or smaller than 0.6) are depicted. Figure 9 shows the network topology of healthy communities. There is a group of highly connected species, including gingivitis- and periodontitis-associated taxa, with *Selenomonas sputigena* and *Selenomonas noxia* acting as the main hubs (species with greater numbers of connections) in this network. This finding is consistent with our previous observation of two community clusters in periodontal health, one of which was enriched for gingivitis-associated and core species.[6,33] Perhaps the co-occurrence of a network of gingivitis- and periodontitis-associated species during health implies that these communities are at greater risk of becoming dysbiotic and therefore this analysis could be useful in the identification of subjects at risk for periodontal breakdown.

Figure 10 shows a similar analysis conducted for gingivitis-associated communities. Here, we observed a greater number of significant correlations than in health. An uncultivated species, *Veillonellaceae* HMT 155, is the main hub connecting mostly gingivitis-associated species and a few periodontitis-associated taxa. Although not shown in Figure 10, we also found a significant correlation between taxa present in less than 50% of subjects, which is worth reporting. When we relaxed this inclusion threshold, we observed a strong positive correlation (r = 0.72) between *P gingivalis* and the uncultivated *Saccharibacteria* (TM7) [G5] HMT 356. TM7 are small bacteria with a genome that only encodes proteins for minimal functions and therefore TM7 depend on the metabolic activities of their host species.[73] It is thus possible that this TM7 phylotype depends on *P gingivalis* for survival.

Figure 11 shows the network topology of periodontitis-associated communities. Here, we observed a group of highly correlated species including health-, gingivitis-, and periodontitis-associated taxa, as well as core species. Interestingly, *Veillonellaceae* HMT 155 is again an important hub in these communities. Also of interest are the correlations seen among different species of

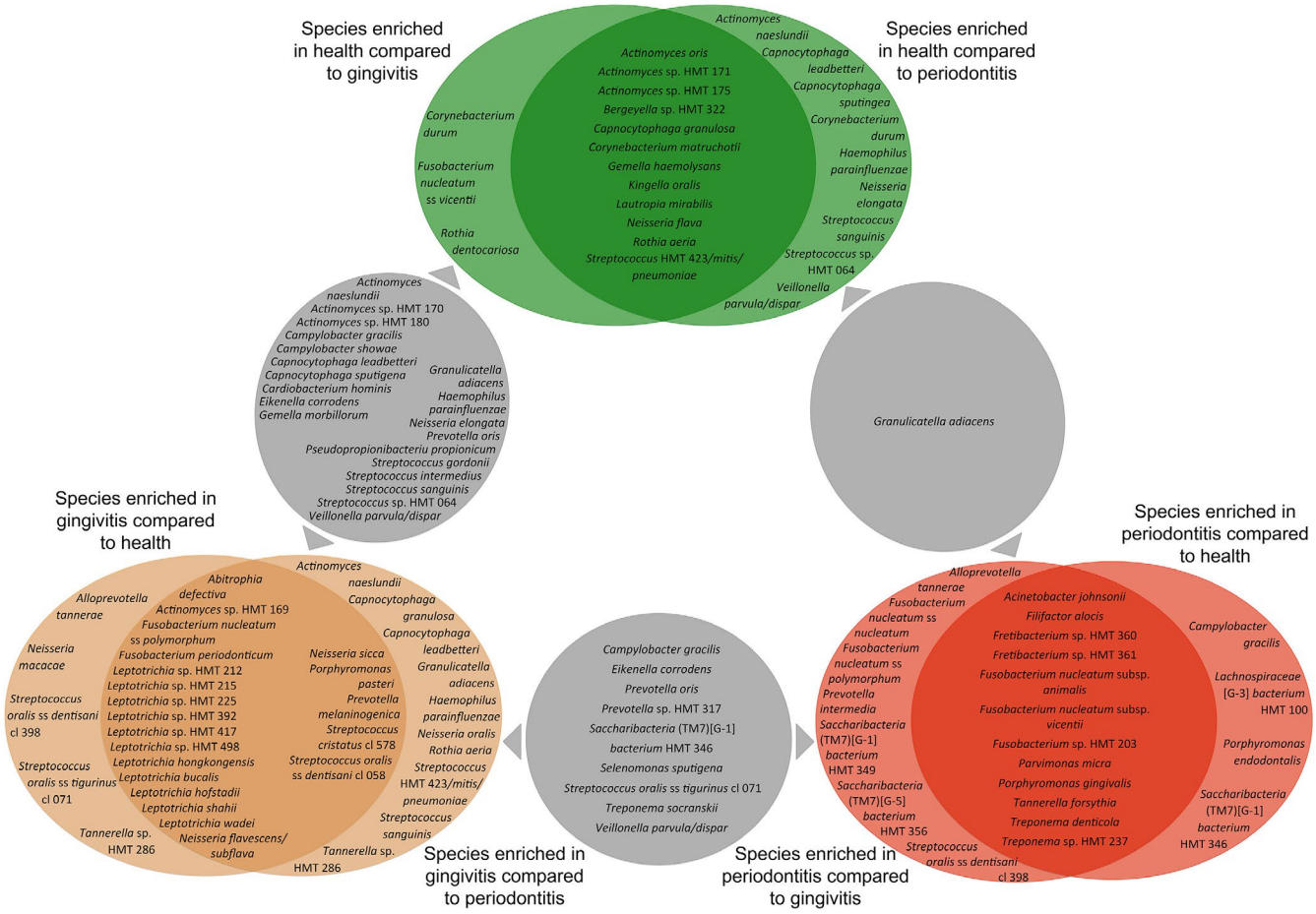

FIGURE 8 Integrated overview of bacterial species that define health, gingivitis, and periodontitis. The 2 green circles (top) contain species that were enriched in health compared with either gingivitis or periodontitis. The area of overlap between the circles contains the species overrepresented in health compared with both gingivitis and periodontitis; and the left and right nonoverlapping segments contain species uniquely enriched in health compared with gingivitis or periodontitis, respectively. In a similar manner, the 2 orange circles (bottom left) contain species enriched in gingivitis compared with health or periodontitis, and the 2 red circles (bottom right) show species enriched in periodontitis compared with health or gingivitis. Enriched bacterial species depicted in circles were selected based on their significant differences in relative abundance, as determined using LEfSe (species shown in Figures 3, 5 and 6). The gray circles show species whose relative abundance did not change when comparing the 2 clinical entities connected by the arrowheads. These unchanged core species were detected in at least 70% of the samples compared

Treponema, and between these *Treponema* spp and *Mogibacterium timidum*. These network correlation analyses can be used as a basis to test interspecies nutritional and metabolic dependencies.

12 | CONCLUDING REMARKS

For the last decade, we have witnessed a surge in our ability to survey the composition of subgingival microbiome communities. These studies have provided a map of the dynamics of the microbiome in relation to the development of periodontal pathology. The focus on the emergence of a few "periodontal pathogens" that provoke pathology has been replaced by the view that periodontitis is initiated by a dysbiotic polymicrobial community, with the subgingival microbiome and the host inflammatory response forming a feedback loop in which the dysbiotic community causes deleterious inflammatory responses and inflammation perpetuates dysbiosis.[34,67,74]

It is clear, however, that the changes in the subgingival microbiome associated with the development of periodontitis are dramatically different from those in gingivitis. Therefore, while inflammation certainly selects for gram-negative proteinase-rich taxa, the emergence of a group of species, including the red complex, *F alocis*, *Fretibacterium* spp, and other taxa indicated in Figure 8, as dominant community members, only occurs in periodontitis. As seen in Figure 7, gingivitis promotes the establishment of periodontitis-associated taxa, allowing them to be detected in most samples; however, periodontitis-associated species are still a very small component of the total microbiome biomass in gingivitis. The emergence of periodontitis-associated taxa as low-abundance species in gingivitis is compatible with the concept that certain low-abundance species with specific virulence factors dysregulate host-protective mechanisms and initiate the events that lead to profound dysbiosis and periodontal tissue destruction.[28] It should be noted, however, that the presence of such "keystone pathogens" may not be

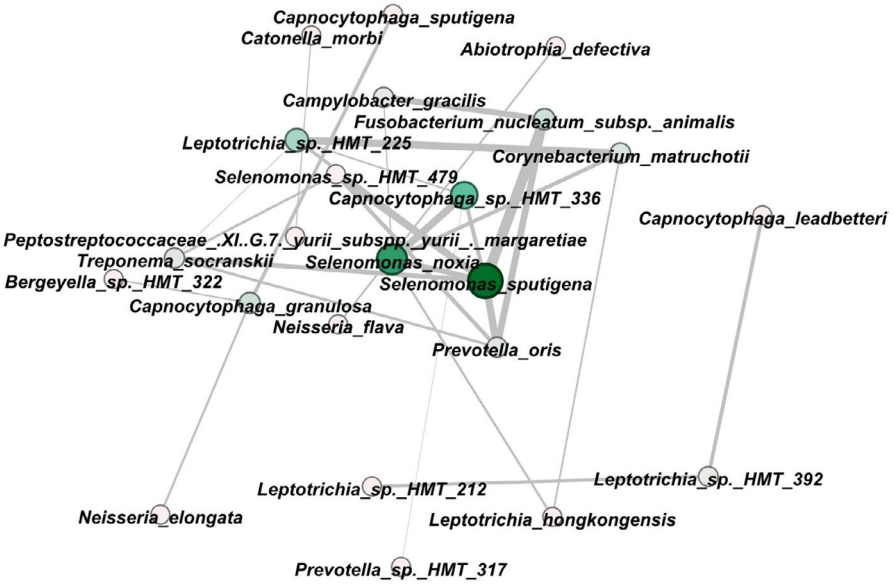

FIGURE 9 Network analysis of bacterial co-occurrence in health-associated communities. Only species detected in at least 50% of samples from healthy subjects were included in the analysis. Each node represents a species. Positive and negative correlations (Spearman) between nodes are depicted as gray or pink lines, respectively. The thickness of lines reflects the correlation coefficient, with thicker lines corresponding to coefficients closer to −1 or 1. Only correlations with a correlation coefficient of <−0.6 or >0.6 are shown. A larger node size and a darker green shade indicate nodes with a higher number of correlations

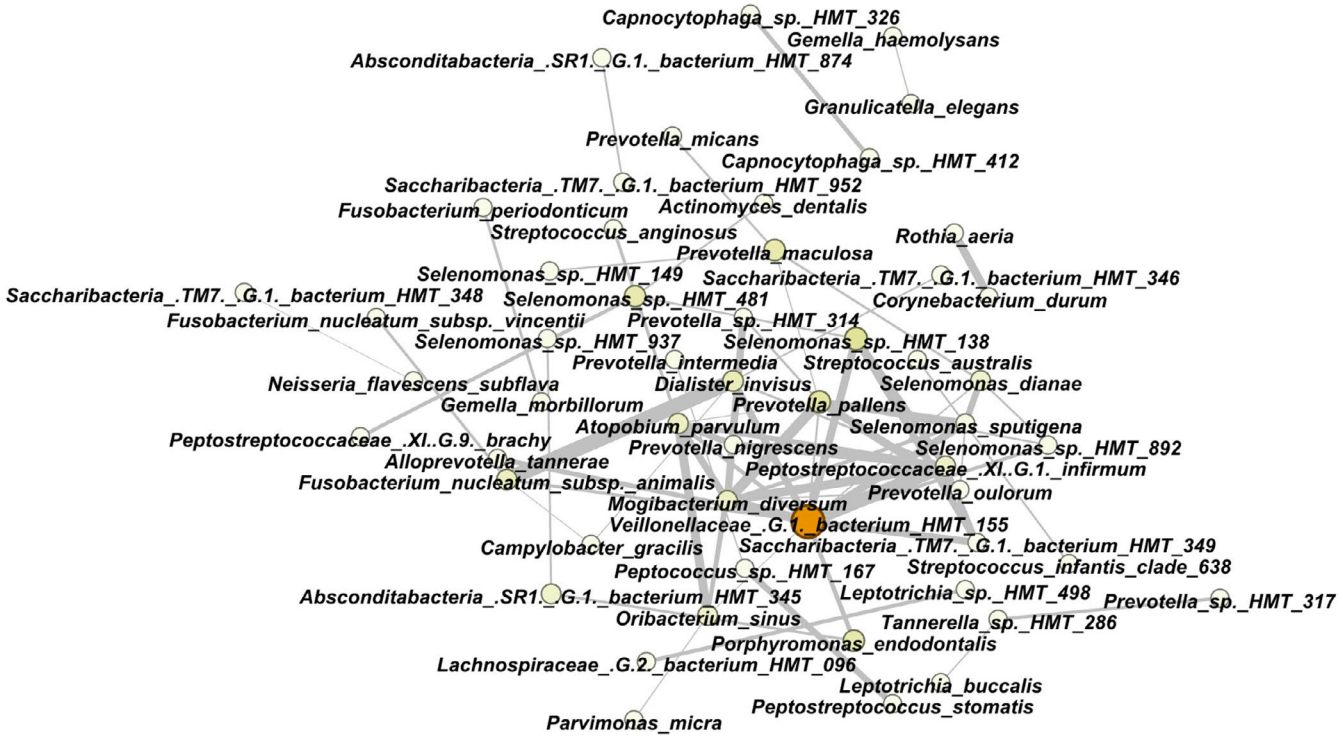

FIGURE 10 Network analysis of bacterial co-occurrence in gingivitis-associated communities. Only species detected in at least 20% of gingivitis samples were included in the analysis. Each node represents a species. Positive and negative correlations (Spearman) between nodes are depicted as gray or pink lines, respectively. The thickness of lines reflects the correlation coefficient, with thicker lines corresponding to coefficients closer to −1 or 1. Only correlations with a correlation coefficient of <−0.5 or >0.5 are shown. A larger node size and a darker orange shade indicate nodes with a higher number of correlations

sufficient for disease initiation, as development of clinical signs of disease is also determined by genetic and environmental determinants, still not fully defined, that modify the host response to the microbiome.[7,46,75]

Although the microbiota is only one side of the equation, a better understanding of the interspecies interactions that lead to the emergence of dysbiotic communities could result in the development of strategies to manipulate the subgingival microbiome, and thereby prevent periodontitis. The overall analysis of species enrichment in health, gingivitis, and periodontitis, and the network co-occurrence patterns shown here, can be used to generate hypotheses for species that may play an important role in maintaining the stability of

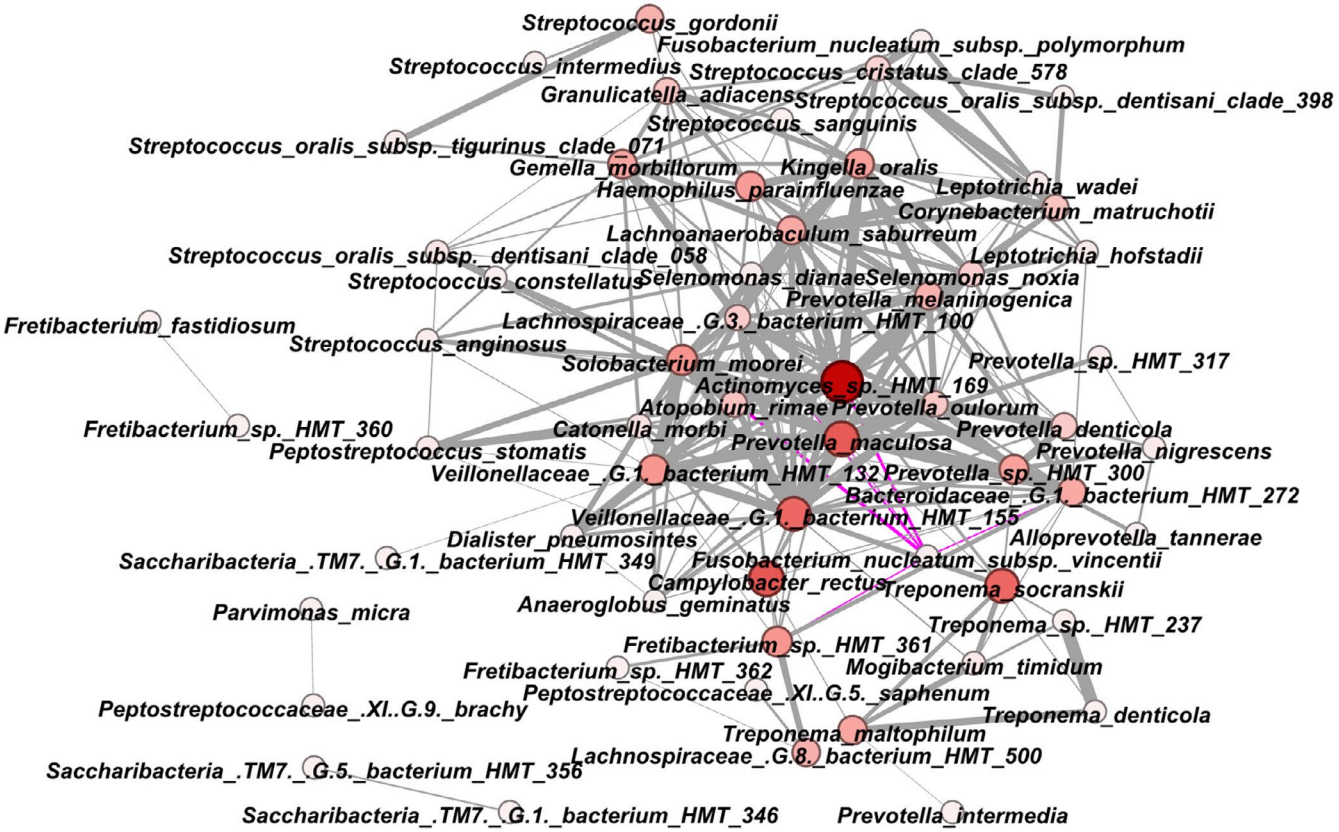

FIGURE 11 Network analysis of bacterial co-occurrence in periodontitis-associated communities. Only species detected in at least 20% of samples from subjects with periodontitis were included in the analysis. Each node represents a species. Positive and negative correlations (Spearman) between nodes are depicted as gray or pink lines, respectively. The thickness of lines reflects the correlation coefficient, with thicker lines corresponding to coefficients closer to −1 or 1. Only correlations with a correlation coefficient of <−0.5 or >0.5 are shown. A larger node size and a darker red shade indicate nodes with a higher number of correlations

communities in each state. Although methods to manipulate oral microbiome communities are still underdeveloped, a few examples are available showing some initial feasibility of targeted elimination of species from a community.[76,77]

These targeted approaches could be directed to species thought to support the metabolic function of communities, such as core species, thereby elucidating their role in promoting the emergence and growth of dysbiotic communities. Our analysis can also be used to interpret the outcomes of different periodontal therapies and establish microbiological goals. The ultimate treatment of periodontitis should aim to restore a health-like community compatible with periodontal stability. Finally, we would like to remark that the current analysis was solely focused on bacteria and undertaken at the taxonomic species level. Subgingival communities harbor Archaea, fungi, and both host- and bacteria-associated viruses,[1] the role of all of which in the overall community function is largely unknown. Moreover, although the ability to analyze these communities at a species level represents progress, a strain-level analysis is needed to understand the role of microorganisms and their specific virulence and metabolic attributes in the development of dysbiosis.

ACKNOWLEDGMENTS

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