The subgingival periodontal microbiota in the aging mouth

Magda FERES[1], **Flavia TELES**[2,3], **Ricardo TELES**[2,3], **Luciene Cristina FIGUEIREDO**[1], and **Marcelo FAVERI**[1]

[1]Department of Periodontology, Dental Research Division, Guarulhos University, Guarulhos, SP, Brazil

[2]Department of Periodontology, UNC School of Dentistry, Chapel Hill, NC, USA

[3]Department of Applied Oral Sciences, The Forsyth Institute, Cambridge, MA, USA

Abstract

Different mechanisms have been hypothesized to explain the increase in prevalence and severity of periodontitis in older adults, including shifts in the periodontal microbiota. However, the actual impact of aging in the composition of subgingival biofilms remains unclear. In the present article, we provide an overview of the composition of the subgingival biofilm in older adults and the potential effects of age on the oral microbiome. In particular, this review covers the following topics: (i) the oral microbiota of an aging mouth, (ii) the effects of age and time on the human oral microbiome, (iii) the potential impact of inflammaging and immunosenescence in the host-oral microbiota interactions, and (iv) the relationship of the aging oral microbiota and Alzheimer's disease. Finally, in order to explore in greater breadth the potential effects of aging on the periodontal microbiota, we present analyses of data compiled from large clinical studies that evaluated the subgingival microbiota of periodontally healthy subjects and periodontitis patients from a wide age spectrum (20–83 years old). Those studies were conducted at Guarulhos University (São Paulo, SP, Brazil) and at The Forsyth Institute (Cambridge, USA), from 1999 to 2014.

The sharp increase in average life expectancy achieved during the 20th century is certainly one of society's greatest accomplishments. Most children born just over 100 years ago did not live past age 50, while currently life expectancy reaches 80 years old in several countries (67). The latest figures from the World Health Organization revealed that in 2010, approximately 524 million people worldwide were aged 65 or older, representing almost 8% of the world's population. This is an unprecedented large population of older individuals, which is expected to triple by 2050, reaching approximately 1.5 billion people (117). One of the major factors contributing to this achievement was the control of infectious and parasitic diseases. While the widespread increase in lifespan is a welcome indicator of socio-

Address for correspondence and reprints: Magda Feres, Centro de Pós-Graduação e Pesquisa-CEPPE, Universidade Guarulhos, 07023-070 Guarulhos, SP, Brazil, mferes@ung.br.

economic progress, it does not come without challenges. Now the leading causes of deaths and illnesses are non-communicable diseases and chronic conditions (67), since there is a wide range of health problems that initiate or worsen with advancing age. Most of these are chronic conditions, which are typically seen with highest frequency in older individuals.

Periodontal diseases, including gingivitis and periodontitis, are the most common chronic infections of adults. In a recent study, published on the journal Lancet, Vos et al. (115) quantified the global burden of non-fatal health outcomes. Periodontal diseases were ranked the #31 among the leading causes of years lived with disability worldwide. Since the prevalence and severity of periodontitis are known to increase with age (3, 57), efforts to optimize the treatment and prevention of those diseases should be a priority both for the scientific community as well as public health policymakers. In the US, the most recent population-based dental survey conducted by the National Health and Nutrition Examination Survey (NHANES) (22) showed that 46% of adults had periodontitis, representing 64.7 million people. Further, the survey revealed that 8.9% of the population presented severe periodontitis, a figure that increased to 11% in subjects aged 65 years and older.

Collectively, the growing world population coupled with worldwide rising life expectancy and the significant decrease in the prevalence of tooth loss throughout the world from 1990 to 2010 (53) are expected to significantly increase the burden posed by periodontal diseases on the individuals and on the healthcare system. Furthermore, the increasing prevalence of several chronic systemic conditions with age, such as cardiovascular and respiratory diseases, as well as diabetes and Alzheimer's disease along with their proposed association with periodontal diseases (5, 37, 70, 83) strongly indicate that the impact of periodontal diseases as a public health problem cannot be overlooked.

Different mechanisms have been hypothesized to explain the increase in prevalence and severity of periodontitis in older adults, including shifts in the periodontal microbiota. However, the actual impact of aging in the composition of subgingival biofilms remains unclear. In the present paper, we will provide a 'state of the art' overview on the composition of the subgingival biofilm of older adults and the potential effects of age on the oral microbiome, based on the most recent literature on the topic. In fact, a review on the topic reveals that most of the previous studies either report on a small number of subjects or utilize low-throughput microbial diagnostic techniques. Thus, in order to explore in greater breadth the potential effects of aging on the periodontal microbiota, in this article we will present analyses of data compiled from large clinical studies that evaluated the subgingival microbiota of periodontally healthy subjects and periodontitis patients from a wide age spectrum (20–83 years old). Those studies were conducted at Guarulhos University (São Paulo, SP, Brazil) and The Forsyth Institute (Cambridge, USA) from 1999 to 2014, and followed very similar protocols for selection of participants, sample collection and microbial analysis.

The oral microbiota of an aging mouth - Current evidence

There is very limited information available in the literature about the periodontal microbiota of older adults (Table 1). The first study to describe the composition of the subgingival

microbiota of older adults was published by Newman et al. in 1978 (68). The authors analyzed subgingival microbial samples from seven periodontally healthy older adults presenting a mean age of 63 years old. They observed a predominance of Gram-positive, aerobic bacteria and relatively small numbers of Gram-negative anaerobes. In addition, Percival et al. (72) studied the effects of the ageing process on the composition of the oral microbiota in a group of 79 dentate periodontally healthy individuals who were subset into four age groups: 20–39, 40–59, 60–79, and >79 years of age. Samples of supragingival biofilm and saliva were cultured on selective and non-selective media. The authors reported no differences among age groups in relation to total counts of bacteria in saliva, as well as for the prevalence of *Streptococcus mutans* and *Spirochaetes* species. On the other hand, *Actinomyces* species, especially *Actinomyces naeslundii* and *Actinomyces oris*, were in statistically significantly higher proportions in the supragingival biofilm of subjects over 60 years of age. Similar results have also been observed by Marsh et al. (59). More recently, Preza et al. (75) described the bacterial diversity of different oral niches from older adults without root caries or periodontitis. Biofilm samples from the tongue dorsum, mucosa of the buccal fold, hard palate, supragingival biofilm from sound root surfaces and subgingival biofilm from the same roots were collected from thirty elderly subjects, with a mean age of 83.9 years (range 73–93), and analyzed using microarrays. The most commonly detected species when all sites were evaluated together were *Streptococcus oralis*, *Veillonella atypica*, *Streptococcus parasanguinis* and *Fusobacterium nucleatum*. The authors suggested that the bacterial profiles of periodontally healthy elders might be more diverse than those of young and middle aged adults.

Only a few studies published in the 1990's evaluated the periodontal microbiota of older adults with periodontitis (11, 20, 78, 82, 86). Rodenburg et al. (78) studied the occurrence of *Porphyromonas gingivalis*, *Prevotella intermedia* and *Aggregatibacter actinomycetemcomitans* in 242 subjects, including 138 individuals with untreated severe periodontitis and 104 with refractory periodontal disease. Pooled subgingival samples were cultured anaerobically. The authors' subset these subjects into four age groups: 14–20, 21–30, 31–40 and 41–70 years of age. They found that the prevalence of subjects colonized by *A. actinomycetemcomitans* appeared to be age related, as it decreased with increasing age. On the other hand, the prevalence of subjects colonized by *P. gingivalis* increased with aging. Other authors have also observed similar correlations between *A. actinomycetemcomitans* and *P. gingivalis* with age (23, 82, 86, 103).

Slots et al. (87) have proposed that older subjects with refractory periodontitis may harbor superinfecting microorganisms. The authors studied the occurrence of subgingival enteric rods, yeasts, *Pseudomonas* and *Staphylococcus* species in 3075 subjects with refractory periodontitis with age ranging from 12 to 93 years old. Older subjects (15%) revealed significantly higher prevalence of enteric rods and *Pseudomonas* species than younger individuals (10.9%). *Staphylococcus* species, on the other hand, showed a higher prevalence in the youngsters compared with older adults. The authors concluded that high levels of subgingival enteric rods and *Pseudomonas* species in some individuals may be important in the pathogenesis of geriatric and other forms of periodontitis and may have therapeutic implications.

Several advances have been made in the microbial techniques used to study the periodontal microbiota since the studies listed above. The newer techniques allowed for increased sensitivity and specificity, as well as the ability to analyze a much greater number of patients and samples, which are important steps toward a better understanding of the periodontal microbiota, including that of older adults. That is crucial step for the development of better strategies for prevention and treatment of periodontitis in these individuals, which may significantly contribute to their oral and systemic health.

The Human Oral Microbiome: the Effects of Age and Time

Recent studies of oral and periodontal microbiology have used sequencing platforms, most commonly employing 16S rRNA –based taxonomic surveys (2, 38, 51). These platforms allow for an open-ended agnostic description of the composition of the microbiomes under study, since they do not target any taxa specifically. However, so far no studies have employed those platforms to specifically investigate the effects of aging in the oral microbiome. Yet, valuable information on the ecological aspects of the oral microbiome, which might provide valuable insights into the influence of aging in the oral microbiome, may be drawn from publications stemming from the Human Microbiome Project (HMP). The HMP is an initiative funded by the National Institutes of Health since 2008 to enable the comprehensive characterization of the human microbiome that shed new light in the importance of the analysis of its role in human health and disease (107).

Given that data regarding the human oral microbiome is lacking, one of the most relevant HMP publications for the topic in question is the study by Yatsunenko et al. (120). In that paper the authors report on the microbial composition of the human gut microbiome viewed across age and geography. The authors evaluated gut microbiome samples from 531 subjects, ranging in age from birth to 83 years old, originating from three distinct geographic locations (USA, Venezuela and Malawi). Their results demonstrated that the phylogenetic composition fluctuated considerably during the first 3 years of life. But, at that age, the bacterial communities evolved towards an adult-like configuration, regardless of the geographic location of the individuals.

Even though it is not known whether the same dynamic microbial volatility is true for the oral cavity, it would not be surprising. After all, major environmental pressures are shaping the developing microbiome as demonstrated by the impact of the mode of delivery (vaginal or cesarian section) (19), the type of feeding (breast or formula feeding (48) and a child's co-habitants and environment (97) in their microbial profiles. It seems that, after an intense albeit brief period of microbial influx and acquisition, humans seem to reach a climax, health-compatible community that seems to be resistant to extrinsic disturbances. Although we do not have direct proof that the same events occur in the oral cavity, and that little changes happen in the oral microbiome from early infancy to old age, indirect evidence exists. For example, Stahringher et al. (97) studied the variability in the microbiome over a decade during adolescence. Eighty-two individuals were sampled more than once, approximately in 5-yr intervals at up to three time-points (12/13, 17/18, and 22/23/24 yr of age). They compared the salivary microbiome of the cohort population of the same age from age 12 to age 17, from age 17 to age 22, and from age 12 to age 22, spanning a period of 5 and

10 yr. After both 5-yr spans, the oral microbiome of an individual resembles itself more closely than that of the population. After 10 yr (12–22), the oral microbiome has a trend toward self-similarity, but this trend was not statistically significant. Therefore, even in the human oral microbiome, where one may anticipate frequent environmental perturbations, there is remarkable stability over long time periods during development up to 5 yr.

Although not controlled for age, the long-term stability of the oral microbiome has been demonstrated in several recent studies. In a benchmark study, Caporaso et al. (12) analyzed tongue biofilms collected daily over a period of 15 months and 6 months, in one female and one male subject, respectively, as well as gut and skin (left and right palm) samples. The authors observed clear body site differentiation as well as individual microbial specificity, which were stable during the observation period. However, their data suggested a surprisingly small temporal 'core human microbiota' (defined as species-level taxa observed across all sampling events) within each individual's body sites, including the oral cavity. When the size of this "core" was assessed, it decreased as follows: mouth > gut > right palm ≈ left palm > across body sites within an individual > across body sites and individuals. The authors hypothesized that this temporal variation may arise from extrinsic factors, including exposure to different types of foods, medications and physical environments (due to travel, for instance) or from intrinsic factors, such as the immune system. It is noteworthy, though, that the oral cavity, which is an open ecosystem exposed to all these factors, showed the greatest compositional temporal stability overtime, in comparison with other body sites.

The extrinsic factors proposed by Caporaso et al. (12) as modulators of the (oral) microbiome were explored in exquisite detail by David et al. (18). The authors addressed an important point: even though disturbances to human microbiota underlie several pathologies, there is currently limited understanding of how lifestyle affects the dynamics of human-associated microbial communities in health. In the most comprehensive time series analysis of the oral microbiome thus far, the authors collected saliva (and fecal) samples daily, from two individuals, during the course of one year. In addition, study participants were equipped with an IPhone and an app to record more than 10,000 longitudinal measurements of human wellness and action, such as fitness, diet, exercise, illnesses and travel, which were ultimately linked to the microbial data. The authors confirmed previous findings regarding the long term compositional stability of the oral microbiome. In agreement with Caporaso et al. (12), the authors also found a relatively small core salivary microbiome, as only a subset of species-level taxa were consistently present over time. Yet, that small consortium dominated the community and comprised 99.7% of the total counted bacteria. It is tempting to speculate that this small consortia comprised the oral microbial species consistently observed in the majority of the classical oral microbiology studies, particularly those known as the "checkerboard panel". The authors also reported that, despite the overall evidence for microbiota stability, certain host actions and health changes led to broad disturbance of the microbial community. For instance, one of the subjects relocated from a major US metropolitan area to Southeast Asia, and thus was exposed to a novel diet and environment. During this period, clear disturbances in community structure were observed, up to the phylum level, revealing increases in *Bacteroidetes* and decreases in *Firmicutes*. Interestingly, the microbiota shifts observed during this subjects' travel reversed upon his return home. One of the key findings in the paper by David et al. (18) is that the stability of the oral

microbiome seems to be quite robust. After all, only a few of the myriad of host factors evaluated could lead to detectable changes in community structure and, in that regard, a change in geographic location and dietary habits was a major influence. It is noteworthy that the findings by David et al. (18) are in accord with those of Haffajee et al. (39). In that benchmark study, the authors demonstrated effect of geographic location on the oral microbiota, as there were some differences in the subgingival microbial profiles of subjects from the US, Sweden, Brazil and Chile.

While the findings of Caporaso et al. (12) and David et al. (18) regarding the stability of the oral microbiome were based on comprehensive time series of daily sample collection of tongue biofilms and saliva samples spanning from 6 to 12 months, respectively, they were obtained from a very small co-hort (1–2 subjects). Yet, they are corroborated by the results from a larger, although less detailed, longitudinal survey of the microbial biogeography of the human microbiome. In that study, Zhou et al. (125) determined the microbial composition of 22 habitats from 279 healthy humans. Those habitats included multiple oral niches (hard palate, keratinized gingiva, buccal mucosa, subgingival biofilm, supragingival biofilm, saliva, tongue dorsum, palatine tonsil, and throat), and other body sites including the anterior nares, skin and vaginal sites as well as stool samples. Samples were collected during 2 time points, and the mean time interval between the two time points was 212 days. The temporal variation analysis, performed using the community similarity between two visits using the Spearman correlation, revealed that, among all sites evaluated, the bacterial communities in oral habitats presented the strongest correlation (>0.6) between first and second visits, particularly tongue dorsum and saliva.

The recent "microbiome revolution" was brought about by democratization of sequencing technologies and has taught and revived many ecological concepts in oral microbiology. It reinforced the notions of microbial community stability, resistance, resilience and core, which are impressive, given the multitude of daily disturbances that human experience from infancy to old age. Yet, the current literature seems insufficient to draw a definitive conclusion about a possible impact of aging on the periodontal microbiota. Most of the reported studies focused on a small number of species and often in a limited number of samples. To the best of our knowledge, no studies to date have comprehensively studied or compared the composition of the subgingival microbiota of older adults with periodontal health or periodontitis. Thus, in order to contribute to this knowledge, the latter portion of this manuscript will present a thorough analysis of the microbial composition of the subgingival biofilm from subjects with periodontal health or disease in different age groups.

Host-microbiota interactions in the oral cavity: the potential impact of Inflammaging and immunosenescence

The oral microbiota is highly complex in organization, holds enormous diversity and have unique capacity to rapid phenotypic changes in response to a wide array of environmental pressures (92, 93, 104). As a whole, it represents a dynamic structure, since it constantly needs to adapt to an ever-changing environment, from birth to old age. Those changes can be elicited by the local milieu, the host immune system and the environment (12, 124). Factors

such as oral hygiene (or lack thereof) (47, 79, 110, 116), smoking (51, 60) and geographic location (18, 39) are examples of local and environmental pressures that can modulate shifts in the oral microbiota, which may also exacerbate host inflammatory response. The balance between the host and microbiota ensures the homeostasis that ultimately leads to oral health. Disturbances in this equilibrium are the basis of the two of the most common infections of the human body, caries and periodontitis (15, 44, 84).

Clearly, the transition from periodontal health to disease is accompanied by a shift from a health-compatible microbiota to a pathogen-enriched community that initiates and maintains the clinical signs of periodontal inflammation and destruction. Interestingly, the benchmarks of the induction and resolution of this process seem to be quite reproducible, as demonstrated by experimental gingivitis studies in different populations and types of patients, including diabetes (80), smokers (81) and older individuals (31, 32). In addition, the microorganisms typically associated with periodontal health and with periodontitis also appear to be a consensus. In recent years, the development and accessibility of microarrays and open-ended, deep-coverage sequencing platforms allowed for the study of the periodontal microbiome in much greater breadth. Yet, along with the identification of new putative pathogens such as *Filifactor alocis* and members of the uncultivated segment of the microbiota, including *Fretibacterium (Synergistetes) species* (55, 56, 73), results from studies using those platforms also identified the "usual suspects", showing members of the orange and red complexes in higher levels in diseases and *Actinomyces sp* and *Streptcococcus sp*, in greater abundance in periodontal health (2, 38, 56, 73, 104). However, despite decades of research in this field, the steps leading to these microbial shifts are not totally understood. Nevertheless, the acquisition or, more likely, the proliferation of periodontal pathogens, individually or in combination, along with changes in the local environment, appears to be essential. In fact, conditions that may interfere with the local or systemic environments, such as smoking, diabetes, genetic polymorphisms and advanced age, are considered risk factors/indicators for periodontal diseases.

The main theories that have been proposed to explain the increased prevalence and/or severity of periodontitis with aging are: i) the cumulative effect of periodontal loss over time (6, 35), ii) alterations in the innate immune and/or inflammatory status (45, 50), and iii) shifts in the composition of the subgingival microbiota (63). The hypotheses ii and iii are greatly interrelated, as changes in the local or systemic immune inflammatory status may influence the oral microbiota and vice-versa (93). It has been postulated that the aging process is associated with a low-grade systemic inflammatory status, even in the absence of clinical signs of infections. This phenomenon, named as inflammaging, increases morbidity and mortality in elders (29) and might interfere with the resident microbial population of the naturally infected areas of the human body, such as the gut and the oral cavity (45, 74). In addition, the decline in function of the immune system brought on by natural age advancement, generally referred to as "immunosenescence" (29, 45, 58, 99), may also contribute to the increased susceptibility of elderly individuals to microbial infections, which may further magnify the effects of inflammaging (30).

Inflammaging and immunosenescense might contribute to the basis of the observed responses of elderly individuals to the periodontal bacterial challenge. Early experimental

gingivitis studies have shown that older subjects responded earlier and more pronounced to biofilm accumulation than younger subjects (49). Besides, they appear to accumulate more biofilm than their younger counterparts, even though the microscopic counts of various types of microorganisms did not reveal any differences between the two groups throughout the period of biofilm accumulation (49). Later studies of similar design (31, 32) confirmed that older individuals accumulated more biofilm and developed more severe gingivitis. They also showed that older individuals presented higher volume of GCF, higher GCF levels of α 2 macroglobulin and IgG3 and larger and more severe inflammatory infiltrate, as determined by gingival biopsies which harbored a higher proportion of B-cells and lower density of neutrophils. Those studies also failed to detect obvious differences between the groups in the composition of biofilm, based on total viable counts and proportions of *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia* and *F. nucleatum ssp*. However, divergent observations were reported by others, in studies that did not observe clinical, immunological or microbial inflammatory differences between young adults and the elderly using the experimental gingivitis model (1, 111, 112, 114, 116).

The aging oral microbiota and its relationship to Alzheimer's disease

The prevailing hypotheses linking oral diseases to systemic conditions posit that periodontal diseases contribute to a systemic inflammatory state and/or the systemic dissemination of oral microorganism, the so-called "mobile microbiome" (46), which may trigger disease processes elsewhere in the body (42). Aging is accompanied by several health problems, including the cognitive decline associated with Alzheimer's disease. Recent epidemiological studies have indicated a possible association between oral health conditions, particularly tooth loss, and decline in cognitive function (34, 66, 71, 98). Research done in the 1990s have demonstrated the elevated presence of spirochetes (including oral treponemes) in the brain of Alzheimer's patients compared to cognitively normal controls (62). Murine models of neurodegeneration have also indicated that mice raised in sterile conditions presented a delayed onset of declined cognitive function (13). It has also been demonstrated that elevated serum tumor necrosis factor-alpha (TNFα) and serum antibodies to *A. actinomycetemcomitans*, *Tannerella forsythia* and *P. gingivalis* were present in Alzheimer's patients compared to controls (52). Interestingly, those authors showed that serum antibodies for these periodontal pathogens had an odds ratio of 6.1 for Alzheimer's disease. After examining data from 2,355 participants in the Third National Health and Nutrition Examination Survey (NHANES-III) who were 60 years of age or older, Noble et al. (69) demonstrated that individuals with the highest serum levels of antibodies to *P. gingivalis* had low cognitive test outcomes. In another study, elevated baseline serum levels of antibodies for *F. nucleatum* and *P. intermedia* also correlated with declined cognitive function 10 years later (96). These studies have led some to propose a mechanism where systemic inflammation, characterized by prolonged exposure to circulating TNFα, would compromise the blood-brain barrier (BBB), allowing bacteria to spread into the brain. In addition, the low-grade systemic inflammation associated with periodontal diseases could contribute to the damage to the BBB. These mechanisms would be compounded by the "immunosenescence" that accompanies aging, potentially leading to an increase in levels of certain members of the oral microbiota and their systemic dissemination. While the adaptive

immune system wanes, innate immunity takes over, contributing to the increased levels of circulating pro-inflammatory cytokines such as TNFα (83).

High-throughput 16S ribosomal RNA gene sequencing has been used to examine differences in the subgingival microbiota in a small group of individuals with and without dementia. Due to the small sample size the results were not statistically significant but the authors reported higher relative abundance of *Fusobacteriaceae* and lower relative abundance of *Prevotellaceae* in subjects without dementia (14). Interestingly, *Fusobaterium species* have been reported to be among the most commonly identified taxa in elderly patients (75, 76). The authors postulated that species from the genera *Fusobacterium* could provide a certain level of protection against cognitive decline by occupying habitats that could be populated by more inflammation-inducing taxa. These recent findings implicating the subgingival microbiota and "immunosenescence" in the cognitive decline that follows Alzheimer's disease highlight the importance of additional studies examining changes in the oral microbiota that occur with aging.

The periodontal microbiota of an aging mouth: a comprehensive evaluation

Study design and subject population

These analyses included data from various cross-sectional and longitudinal studies conducted at the Center for Clinical Research at Guarulhos University (São Paulo, Brazil) and at The Forsyth Institute (Cambridge, USA), from 2002 to 2014 and 1999 to 2014, respectively. Data from 1330 subjects, being 1084 with periodontitis and 246 with periodontal health, were evaluated. The clinical data included probing depth (PD) and clinical attachment level (CAL), which were recorded at six sites per tooth, from all teeth. Subgingival biofilm samples were taken from nine sites per subject at each experimental time point, 3 in each of the following PD categories: 3 mm (shallow), 4–6 mm (intermediate) and 7 mm (deep). The samples were analyzed for their content of 40 bacterial species using checkerboard DNA–DNA hybridization (61, 94). A total of 10.568 biofilm samples and 422.720 data points were evaluated.

Included subjects were at least 20 years old and were in good general health. Subjects with periodontitis had a minimum of 15 natural teeth and four teeth with at least one site each with PD and CAL 5 mm. Periodontally healthy individuals had a minimum of 20 teeth, no sites with PD or CAL >3 mm, and <20% of the sites with bleeding on probing. Exclusion criteria were pregnancy, nursing, periodontal therapy and antibiotic administration within the previous 3 months, any systemic condition that might have affected the progression of periodontitis and need of antibiotic coverage for routine periodontal procedures. No subjects with localized aggressive periodontitis were included in the study.

Table 2 presents the baseline demographic and clinical characteristics of the population, discriminated by center. The significance of differences between the two databases in each clinical group was assessed using the Student T test. Subjects ranged in age from 20 to 83 years. Subjects with periodontitis and periodontal health from Guarulhos University presented higher mean full-mouth PD and CAL than those from the Forsyth Institute (p<0.000). In addition, the Forsyth database had more smokers in the periodontally healthy

group, and their periodontitis group had a higher mean age than that of Guarulhos University (p<0.000). No differences were observed between the two databases for gender.

Subjects with periodontitis and periodontal health from both centers were grouped and categorized according to three age groups as follows: <35 (young), 35–64 (adults) and >64 (older adults) years old. Although the population included in this study was not rigorously categorized in Chronic and Aggressive Periodontitis, according to the standard periodontal disease classification of the AAP (4), most of the individuals in the young group presented Aggressive Periodontitis, while those in the other two groups (adults and older adults) had exclusively Chronic Periodontitis. The demographical and clinical characteristics of the full database, stratified according to the three age groups are presented in Table 3. The significance of differences among groups was assessed using analysis of covariance (ANCOVA) adjusted for smoking, gender, baseline PD and geographic population. The significance of differences between pairs of comparisons was determined using Tukey's multiple comparison test. As expected, the mean age differed significantly among the three groups, as well as the percentage of smokers, which was lower in the young group with periodontitis (p<0.05). Mean PD did not differ among age groups, but older adults with periodontal health or disease had a statistically significantly higher mean CAL in comparison with the young and adult groups.

In order to test the hypothesis that "older adults with chronic periodontitis present a subgingival microbiota distinct of that observed in younger individuals" we have formulated a series of questions that will be discussed in the following sections.

Does aging influence the subgingival microbiota associated with periodontal health or disease?

With the aim of addressing the influence of aging in the subgingival microbiota, we evaluated the mean counts and proportions of 40 bacterial species in the subgingival biofilm samples of subjects with periodontal health or periodontitis in each age group and the results are depicted in Figs 1 and 2. Figure 1 presents the results for the periodontally healthy individuals. No statistically significant differences were observed in the levels or proportions of the bacterial species evaluated between the older adults and the other group of adults or the youngsters. Although not statistically significant, it is worth mentioning that older adults showed a trend towards higher levels of the three *F. nucleatum* subspecies. This finding is in accord with the results from Preza et al. (75, 76), who described the high prevalence of *F. nucleatum* subsp. *polymorphum* oral taxon 202 in a group of elderly subjects (age range 73–93), associated particularly with healthy root surfaces (75). The presence of gingival recession and root surface exposure in the older group of our study might help justify this finding. Interestingly, using 16S bacterial ribosomal RNA gene sequencing, Cockburn et al. (14) found higher relative proportions of *Fusobacterium* species in subgingival biofilm samples from subjects without dementia compared to individuals with declined cognitive function. They proposed that species form the genus *Fusobacterium* could provide a certain level of protection against dementia by filling the subgingival habitat, warding off the colonization of more inflammation-inducing bacterial species.

Figure 2 display the results for subjects with periodontal disease. It was noted that the levels of two red complex pathogens, *P. gingivalis* and *T. forsythia*, were elevated in the young group in comparison with the other two groups (p<0.05), and two species normally associated with periodontal health, *A. naeslundii* and *A. oris*, were elevated in the older adults in comparison with youngsters and adults (p<0.05). This same trend was observed when the proportions of the microbial complexes were evaluated. The young group had higher proportions of the red complex pathogens in comparison with the other two groups (p<0.05). Red complex pathogens accounted for 25.9% of the 40 species evaluated in the youngsters, 19.2% in the adults and 17.5% in older adults. On the other hand, older adults harbored the highest mean proportions of the microbial group comprised of four *Actinomyces* species: *Actinomyces gerencseriae*, *Actinomyces israelii*, *A. naeslundii* and *A. oris* (p<0.05). Also, the youngsters presented higher proportions *A. actinomycetemcomitans* in comparison with the other two groups (p<0.05).

The next analysis provides a comparison of the subgingival microbial composition of subjects with periodontal health and disease within each age group (Fig. 3). Overall, the differences between health and periodontitis for the proportions of the 40 bacterial species evaluated were very similar among the three age groups. The proportions of the three red complex pathogens, *Eubacterium nodatum* and *P. intermedia* were statistically significantly elevated in the periodontitis subjects of the older adults, adults and young groups, while *A. naeslundii* and *A. oris* were elevated in health. A few other species compatible with periodontal health were also elevated in the samples from periodontally healthy individuals in comparison with those with periodontitis, such as *Actinomyces odontolyticus* and *Veillonella parvula* in the young group, *Capnocytophaga gingivalis* in the young and adult groups, and *Streptococcus sanguinis* in the older adults group.

The overall evaluation of the results described above suggest that the main difference observed between the composition of the subgingival microbiota of the older adults and the two other age groups was higher levels and proportions of the *Actinomyces* species in the group of older individuals (p<0.05). The actual significance of this information is not totally understood, since it seems contradictory to the higher prevalence and severity of periodontitis observed in these subjects, as *Actinomyces* are healthy associated species (2, 89, 90, 104, 106). It could be speculated that the higher diversity of surfaces found in the oral cavity of older individuals, in comparison to younger individuals, could favor the overgrowth of *Actinomyces*, which are considered important biofilm formers due to their good adherence properties (100, 113). Therefore, this species might have a selective advantage, over other species, in colonizing the wide variety of surfaces found in the oral cavity of older adults. Previous studies have pointed out *Actinomyces* as a main genus of the predominant cultivable microbiota of dentures from patients with a healthy mucosa or stomatitis (108, 109). In addition, it has been shown that biofilms on tooth surfaces may harbor higher proportions of *Actinomyces* compared to soft tissue surfaces (93). Older subjects normally present numerous exposed root surfaces, increasing the area for the development of tooth-associated biofilms. This might have been the case of the older adults group in the present study, which presented greater mean full-mouth CAL than PD, indicating a high number of recessions. Percival et al. (72) reported that *Actinomyces* species were in statistically significantly higher proportions in the supragingival biofilm of

subjects older than 60 years of age. In accord with these findings, Preza et al. (75, 76) reported that *Actinomyces* species were commonly found in supragingival biofilms adjacent to healthy root surfaces (75). Further, *Actinomyces* have also been demonstrated to be associated with healthy and carious root surfaces in previous studies (122).

Another interesting observation in this evaluation was the higher levels and proportions of *A. actinomycetemcomitans* and red complex species in the young group (mostly composed of aggressive periodontitis) in comparison with the other two groups of adults with periodontitis (chronic periodontitis). The finding of higher proportions of *A. actinomycetemcomitans* in the youngsters is in accord with previous studies in the literature that suggested an important role of this microorganism in the etiology of aggressive periodontitis in young individuals (23, 28, 36, 64, 123). On the other hand, this species seems to have a lower influence on the onset and progression of periodontitis in adults, as previously suggested (23, 78), or in older adults, as shown in the present study. The highest counts and proportions of red complex pathogens in the young group is also in agreement with previous studies that have indicated a possible role of red and orange complex pathogens in the etiology of aggressive periodontitis (16, 23, 33, 36, 54, 101, 119).

Does aging influence the microbial composition of sites with different PDs or of subjects with different extent and severity of disease?

The next question asked was whether aging would influence the microbiota of specific sites or subjects with different severity of disease. Since we did not find main differences between the subgingival microbial profile of the older adults and the other two age groups, we wanted to assess if there were any differences that could be related to a specific category of sites, as the depth of the pocket may influence the local environment and consequently the microbiota. For this analysis we compared the microbial profiles of the three age groups according to different PD categories: shallow, intermediate and deep. These results (Fig. 4) were very similar to those observed when all sites were analyzed together (Fig. 2), namely higher levels of *Actinomyces* species in the older adults group. Interestingly, these species seemed to be quite well distributed in the oral cavity of the older subjects, as they were statistically significantly elevated in the shallow, intermediate and deep pockets, in comparison with the youngsters and the adults. On the other hand, the red complex species and *A. actinomycetemcomitans* were statistically significantly elevated in the young group only in the shallow sites. This information might have ecological implications, as it might indicate an important role of these pathogens in initiating the disease process, and not only as merely accessory pathogens that grow in levels and proportions favored by the inflammatory environment associated with periodontitis.

We also wanted to access any possible difference between the microbial profiles of the different age groups that might have been hidden by the various degree of disease severity of the subjects from each age group. Thus, we subset the subjects according to their disease severity, as follows: i) "the least" severe subgroup: subjects presenting mean PD below the lower quartile of their age group, and ii) "the most" severe subgroup: subjects presenting mean PD above the upper quartile of their age group. The subgroups were compared for the levels of the 40 species evaluated and the results are presented in Fig. 5. Overall, the

individuals with most severe disease in each age group had statistically significantly higher levels of red complex pathogens and of a few orange complex species. The young subjects with severe disease individuals also presented statistically significantly higher levels of two *Actinomyces* and three *Streptococcus* species in comparison with the youngsters from the least severe subgroup. Two *Actinomyces* species and two *Capnocytophaga* species were, respectively, elevated in the subgroups of adults and older adults with severe disease. We also compared the three age groups according to the two disease severity categories (Fig. 6). The comparison among the most severe subgroups showed the same trend observed previously, when all subjects from each age group were compared (Fig. 2), which was higher levels of *Actinomyces* in the older adults group and higher levels of red complex species in the young group, but these differences were not statistically significant. The smaller sample size in the subgroups might have influenced the lack of statistical significance in the present analysis.

Concluding remarks

The proportion of older adults has increased substantially worldwide in the past decades, and the demands of this growing segment of the population have been influencing science, technology and the health services. The prevention and treatment of oral and systemic health problems in older adults represent a new challenge for researchers and clinicians. Periodontology is one of the specialties of dentistry mostly affected by this new reality, as novel theories suggest the existence of a complex interplay between the immune system of older adults and the microbiota of naturally infected areas of the body, such as the gut and the oral cavity (8, 45, 120). There is growing evidence that the low-grade, chronic, systemic inflammation observed in aging (inflammaging) is an important risk factor for both morbidity and mortality in the elderly people (30), and inflammaging may be potentiated by products produced by the microbial communities existent in the human body, such as the periodontal or gut microbiota, which can reach the circulation (7, 8, 85). Indeed, there is also evidence that periodontitis is associated with elevated levels of systemic pro-inflammatory biomarkers (65, 102) especially in older adults (10). Thus, a deep understanding about the onset, progression and treatment of periodontitis in older adults is crucial for improving the oral and systemic health of these individuals.

One of the theories that has been considered to explain the higher prevalence and/or severity of periodontitis in older adults is a different subgingival microbial profile. However, it is quite surprising to observe that no studies to date have comprehensively evaluated the periodontal microbiota of older adults, and no association studies have directly compared the subgingival microbial profile of older adults with that of periodontal health or disease. Although the majority of the association studies have included older adults in the periodontitis groups, most of the periodontally healthy subjects were younger. Thus, healthy and periodontitis subjects are normally not matched by age in these studies, hampering definitive conclusions about the microbiota associated with periodontal health or disease in older individuals (63).

The analyses presented in this article aimed to provide information about the composition of the subgingival biofilm of older adults, aged 64 years or more, by evaluating data from 1330

subjects with periodontal health or periodontitis from different age groups. It should be underlined that the present study has limitations. First, as most studies that investigated the impact of aging in the local microbiome, it is not a true longitudinal study. Rather it presents a comparison of the snapshots of three different groups of individuals, subset according to randomly determined age brackets. An ideal comparison of the effects of aging in the periodontal microbiome should follow a cohort of individuals over time, where each individual is its own control. However, one can appreciate the huge logistical challenges imposed by such design. Yet, the relevance of these types of analyses highlight the need to create large, curated and standardized biorepository of oral (and other types of) samples from birth cohorts.

Second, the combination of clinical and microbiological data from different research centers or from different studies within each center may present certain constrains. The inclusion criteria may have varied slightly among the various studies, the examiners were not calibrated and there were statistically significant differences between the two centers in terms of the baseline clinical and demographic features. The analyses were adjusted for these differences by using age, gender, mean PD, mean CAL, geographic location and smoking status as covariates.

Third, regarding the microbiological evaluation, this study presents results for the 40 bacterial species proposed by Socransky et al. (89, 92) as the main microorganisms associated with either periodontal health or disease. It is well recognized that the periodontal microbiome encompasses considerably more taxa than those included in this "small" group of bacterial species (21). Nevertheless, this panel of species has been successfully used as a biological marker for studies of periodontal disease risk and treatment in both research centers (23, 25, 26, 27, 40, 41, 88, 91, 95, 104, 106) and by other groups of investigators worldwide for over 20 years (16, 17, 118, 119). Furthermore, many recent studies that have used more comprehensive techniques, such as microarrays (104) and 16S rRNA sequencing (2, 38, 125) to study the oral microbiome have, in general, supported many of the results obtained in earlier studies using checkerboard DNA-DNA hybridization.

In spite of the limitations described above, the present study provides the first comprehensive evaluation of the periodontal microbiota associated with periodontal health or periodontitis in older adults. Overall, the data does not support a substantial influence of aging in the composition of the subgingival microbiota, which is corroborated by a number of earlier studies using the experimental gingivitis model and indirectly supported by recent studies demonstrating the long-term stability of the oral microbiome (1, 12, 18, 31, 49, 97, 116).

The data of the present study suggested that the composition of the subgingival biofilms of older adults with periodontal health and periodontitis is very similar to that of youngsters and adults. In addition, the association study comparing the subgingival biofilm of healthy and periodontitis subjects in the different age groups supported the notion that the already known periodontal pathogens were elevated in disease, in the three groups. The only difference observed in all the analyses, between the microbial composition of older adults and the other two age groups, was higher levels and proportions of *Actinomyces* in the older

subjects. This might be explained by the higher diversity of surfaces and a higher prevalence of exposed roots in these subjects, as *Actinomyces* species may present a selective advantage over other oral species to colonize the supragingival plaque and prosthesis surfaces. Nonetheless, *Actinomyces* are considered host-compatible species, and their elevated levels and proportion in older patients might not have major impact in treatment response. Also, when the groups were subset into different severities of disease, the subgingival microbial composition of the older adults showed a similar pattern of that observed in youngsters or adults, which was more strict anaerobe pathogens in subjects with the highest disease severity. Likewise, aging did not seem to influence the microbiological profile of sites with different PDs.

Although the data of this study did not indicate major differences between the subgingival biofilm composition between older adults and the other age groups for the 40 bacterial species evaluated, it is possible that other species of bacteria that were not detected by these DNA probes could have an important role in the disease process in a specific age group. However, in agreement with our data, the results of recent studies using open-ended approaches, which in theory can identify any taxon present in the sample under study also failed to indicate a major role for aging as a determinant of the oral microbiome (12, 18). What is possible though, and has never been investigated, is that the activity of the oral microbiome might be influenced by age. The potential importance of function analysis in the study of the impact of aging in the oral microbiome is supported by two recent benchmark studies evaluating the metatranscriptomics of subgingival biofilm in periodontal health and disease (21) and during periodontal disease progression (121). Those types of studies have opened new venues for the understanding of the mechanisms used by oral bacteria to induce periodontal breakdown and might do the same for the aging process. Thus the oral microbiome transcriptome should be further explored during the process of aging in order to expand the knowledge in this field. For instance, using metagenome function analysis of the gut microbiome, Rampelli et al. (77) recently were able to distinguish groups of young adults, elderly and centenarians, presenting mean age of 30, 70 and 100 years old. It should also be highlighted that their peculiar study population (including individuals over 100 years of age) might have contributed to their ability of find such differences, because the majority of the previous studies did not include a group of extreme aging. Likewise, studying young adults, elderly and centenarians, recently, Biagi et al. (9) showed that the (extreme) ageing process affects the structure of the human gut microbiota, as well as its homeostasis with the host's immune system. The above-mentioned studies indicate that the centenarians might offer valuable insights that can only be observed in the extremes of the lifespan, and this would be an important group of subjects for future studies.

Finally, future studies should also focus on the interplay between periodontal pathogens/ periodontal destruction and the host/immune-response of older individuals, as well as in the most effective preventive and treatment protocols for these subjects. It must be bear in mind that the same infectious burden in an older adult may have worse consequences than in a younger individual. As mentioned before, periodontitis may influence inflammaging, inflammaging may influence periodontitis, or still, the two processes may reinforce and potentiate each other (45). This may have harmful consequences for the patient general health, since inflammaging may increase mortality in older individuals. Indeed, some studies

have associated specific segments of the oral microbiota with systemic problems, including dementia and Alzheimer's disease (14, 83, 124). Therefore, the knowledge of how aging as well as inflammaging influence the microbiomes of older adults could greatly benefit the overall health of these individuals.

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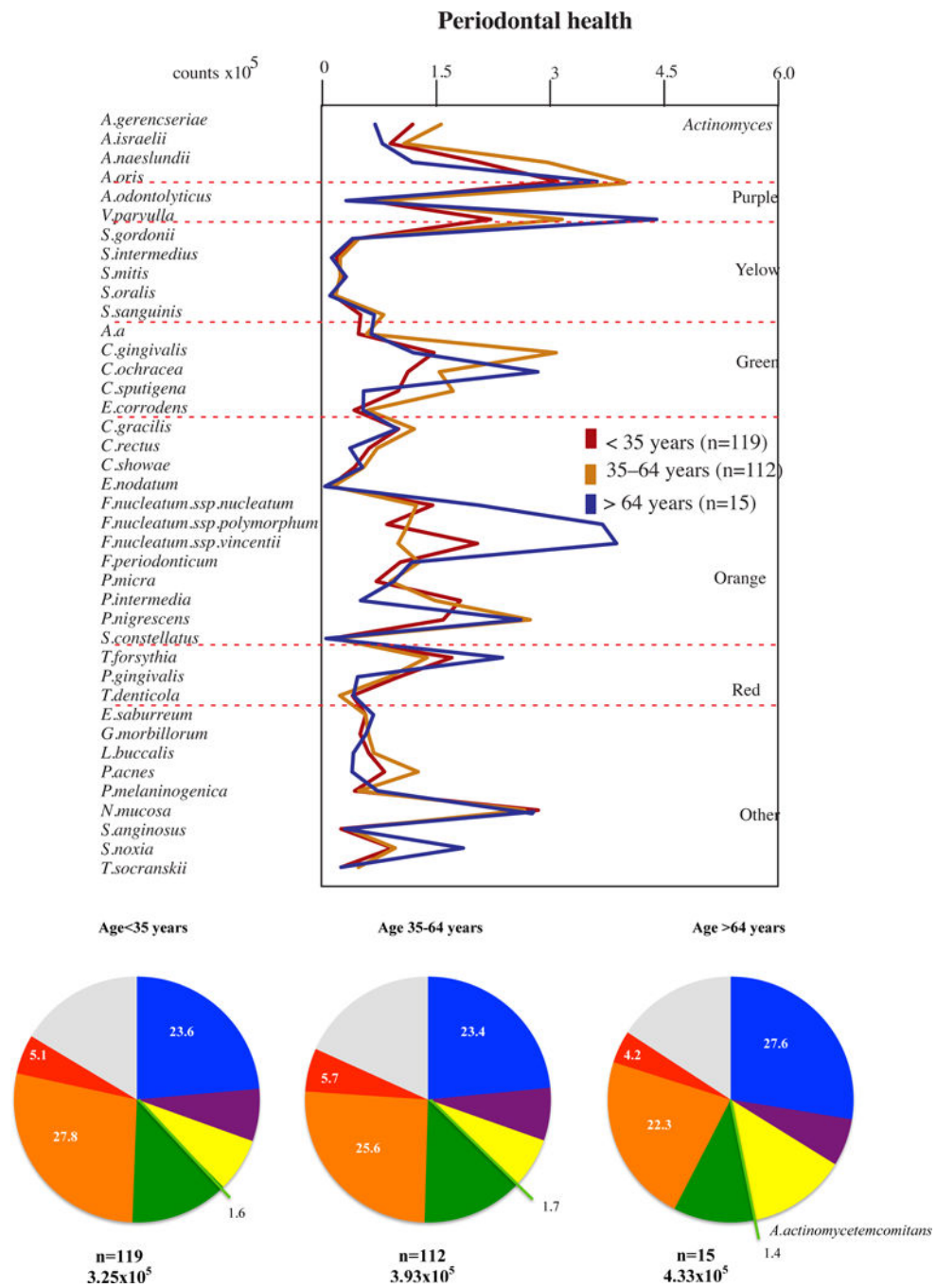
Periodontal health

Figure 1.
Profiles of the mean counts ($\times 10^5$) and of the mean proportions of 40 taxa in subgingival biofilm samples taken from 246 subjects with periodontal health subset into three age groups (<35, 35–64 and >64 years of old). Nine subgingival biofilm samples were taken from each subject and were analyzed separately to determine their content of the 40 species of bacteria. The species were ordered and grouped according to the microbial complexes described by Socransky et al. [89]. The mean values for each species were averaged within a subject and then across subjects in each age group. The colors in the pie charts represent the different

complexes. *Actinomyces* spp. are represented in blue and *A. actinomycetemcomitans* in light green. The grey color represents species that did not fall into any complex. The significance of differences among groups was determined using analysis of covariance (ANCOVA), adjusted for smoking, gender, baseline probing depth and clinical attachment level and geographic population. The significance of differences between pairs of comparisons was determined using Tukey's multiple comparison test (different letters indicate significant differences between age groups).

Figure 2.
Profiles of the mean counts ($\times 10^5$) of 40 taxa in subgingival biofilm samples taken from 1084 subjects with periodontitis subset into three age groups (<35, 35–64 and >64 years of old). Nine subgingival biofilm samples were taken from each subject and were analyzed separately to determine their content of the 40 species of bacteria. The species were ordered and grouped according to the microbial complexes described by Socransky et al. [89]. The mean values for each species were averaged within a subject and then across subjects in each age group. The colors in the pie charts represent the different complexes. *Actinomyces* spp.

are represented in blue and *A. actinomycetemcomitans* in light green. The grey color represents species that did not fall into any complex. The significance of differences among age groups was determined using analysis of covariance (ANCOVA), adjusted for smoking, gender, baseline probing depth and clinical attachment level and geographic population. The significance of differences between pairs of comparisons was determined using Tukey's multiple comparison test (different letters indicate significant differences between age groups).
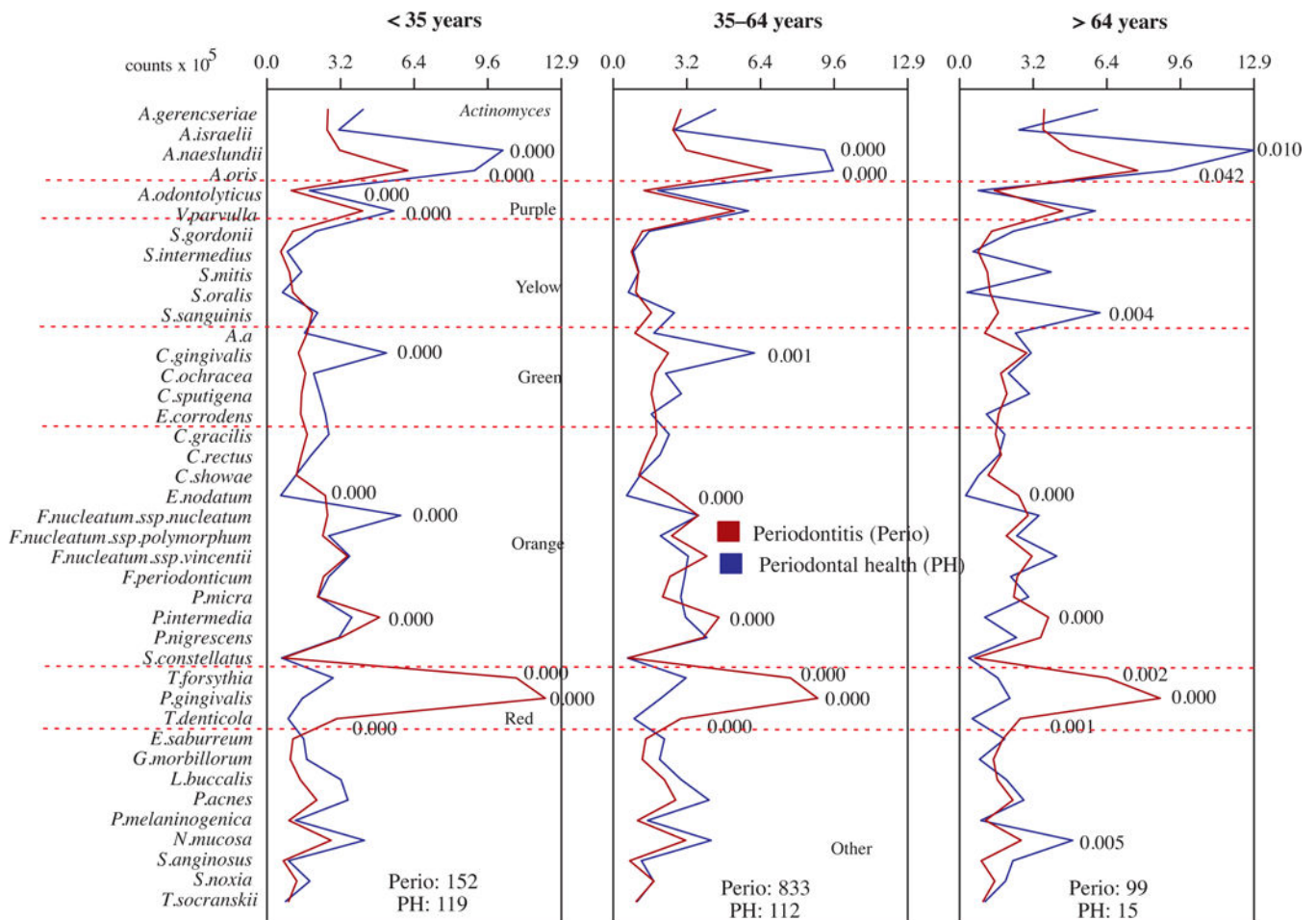
Figure 3.
Profiles of the mean counts ($\times 10^5$) of 40 taxa in subgingival biofilm samples taken from 1084 subjects with periodontitis (Perio) and 246 periodontally healthy subjects subset into three age groups (<35, 35–64 and >64 years of old). Nine subgingival biofilm samples were taken from each subject and were analyzed separately to determine their content of the 40 species of bacteria. The species were ordered and grouped according to the microbial complexes described by Socransky et al. [89]. The mean values for each species were averaged within a subject and then across subjects in the two clinical groups for each age category. The significance of differences between clinical groups was determined using analysis of covariance (ANCOVA), adjusted for smoking, gender, baseline probing depth and clinical attachment level and geographic population.
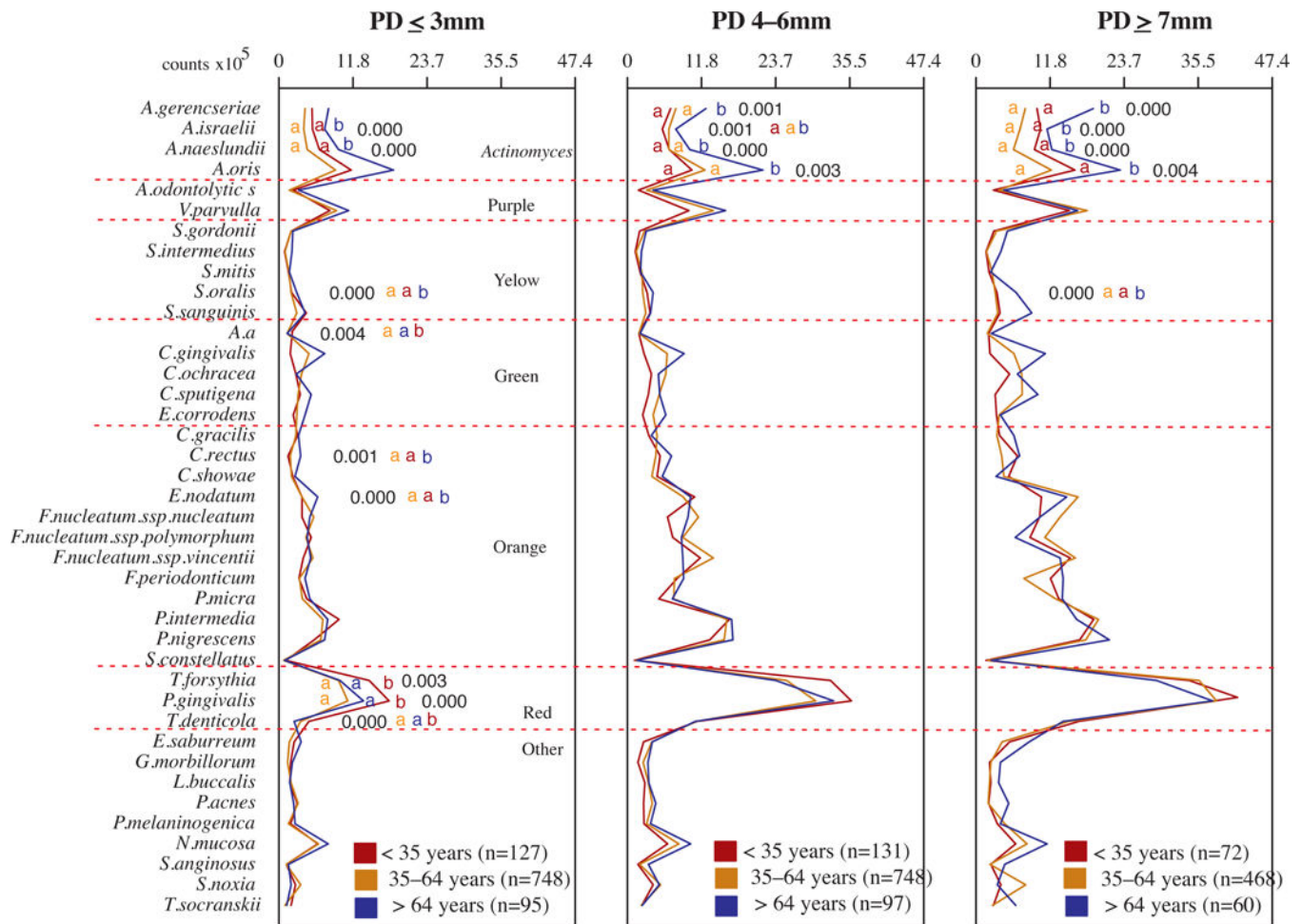
Figure 4.
Profiles of the mean counts ($\times 10^5$) of 40 taxa in subgingival biofilm samples taken from 1084 subjects with periodontitis subset into shallow (probing depth (PD) 3mm), intermediate (PD 4–6mm) and deep (PD 7mm) sites in different age categories (<35, 35–64 and >64 years of old). Three subgingival biofilm samples of each PD category were taken from each subject and were analyzed separately to determine their content of the 40 species of bacteria. The species were ordered and grouped according to the microbial complexes described by Socransky et al. [89]. The mean values for each species in each PD category were averaged within a subject and then across subjects in the three age groups. The significance of differences among age categories was determined using analysis of covariance (ANCOVA), adjusted for smoking, gender and geographic population. The significance of differences between pairs of comparisons was determined using Tukey's multiple comparison test (different letters indicate significant differences between age groups).
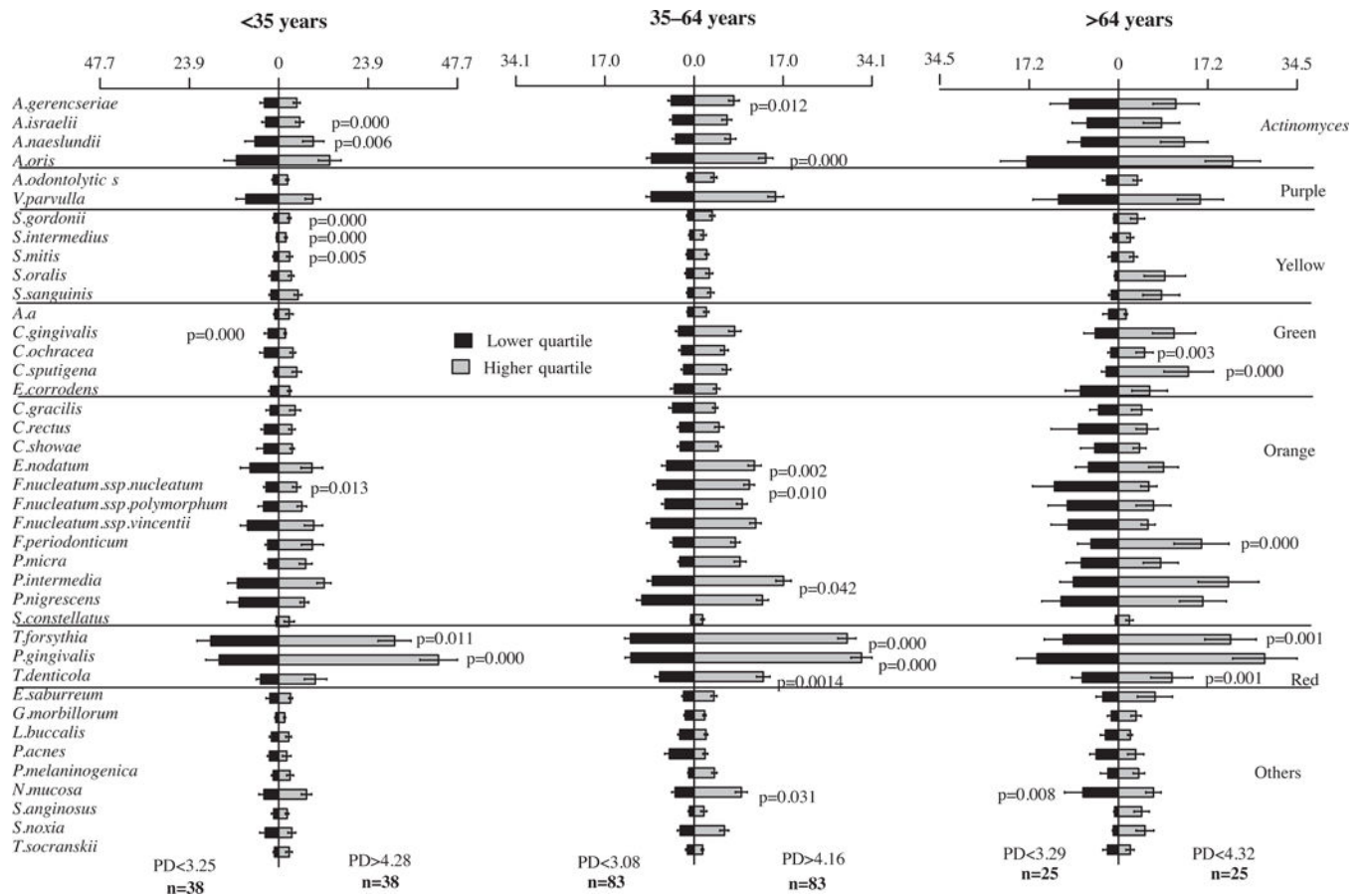
Figure 5.
Bars charts (± standard error of the mean) of the mean counts ($\times 10^5$) of 40 taxa in subgingival biofilm samples taken from 292 subjects with periodontitis subset according to two severity categories ("the least" severe and "the most" severe) in different age groups (<35, 35–64 and >64 years old). Nine subgingival biofilm samples were taken from each subject and were analyzed separately to determine their content of 40 species of bacteria. The species were ordered and grouped according to the microbial complexes described by Socransky et al. [89]. The mean values for each species were averaged within a subject and then across subjects. The significance of differences between severity category in each age group was determined using analysis of covariance (ANCOVA), adjusted for smoking, gender and geographic population.
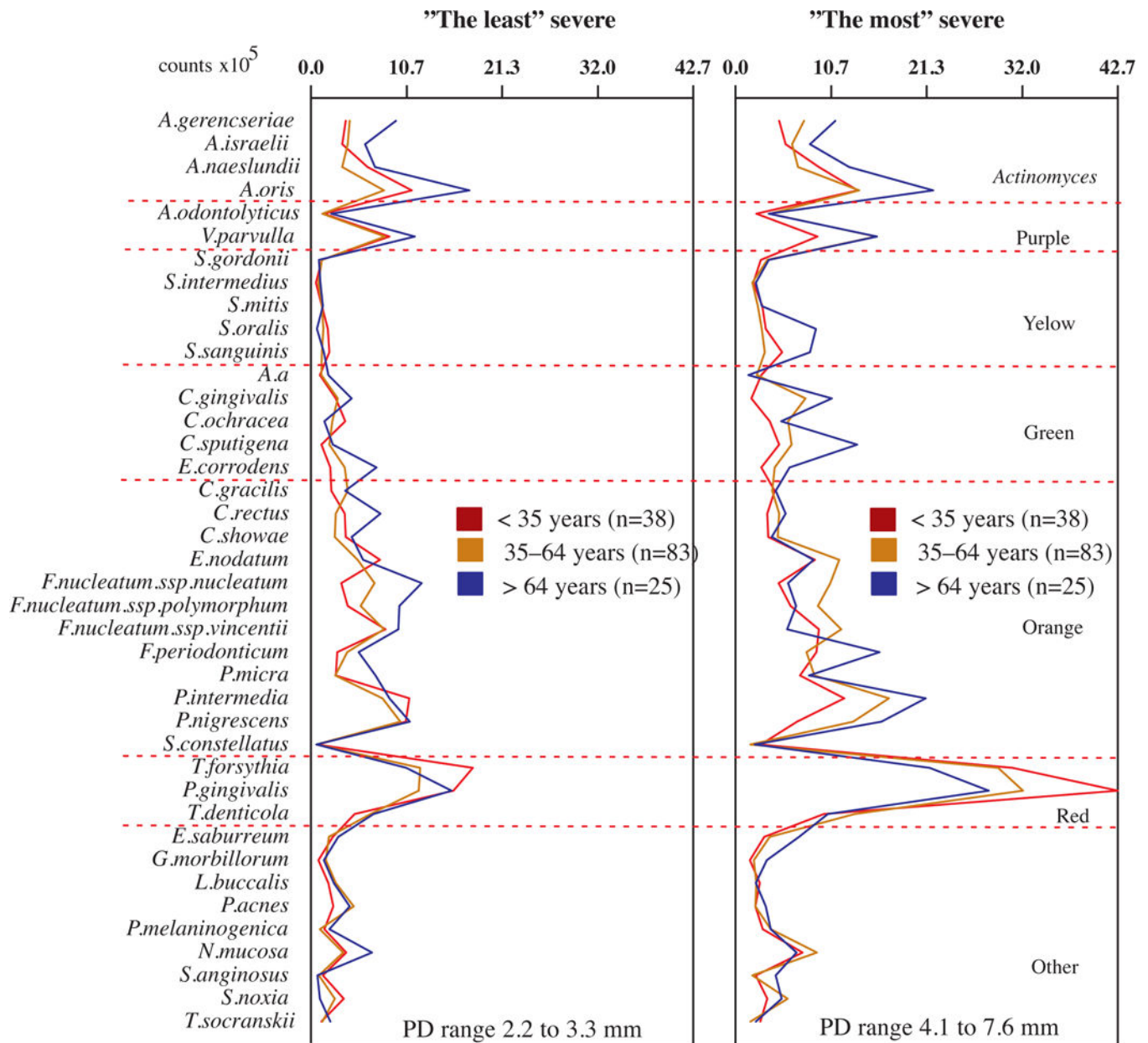
Figure 6.
Profiles of the mean counts ($\times 10^5$) of 40 taxa in subgingival biofilm samples taken from 292 subjects with periodontitis subset into three age groups (<35, 35–64 and >64 years old) in different severity categories ("the least" severe and "the most" severe). Nine subgingival biofilm samples were taken from each subject and were analyzed separately to determine their content of 40 species of bacteria. The species were ordered and grouped according to the microbial complexes described by Socransky et al. [89]. The mean values for each species were averaged within a subject and then across subjects in each severity categories. The significance of differences among age groups into each severity category was determined using analysis of covariance (ANCOVA), adjusted for smoking, gender, baseline probing depth and geographic population. The significance of differences between pairs of

comparisons was determined using Tukey's multiple comparison test (different letters indicate significant differences between age groups).

Table 1

Studies evaluating the oral/periodontal microbiota of older adults or that have compared the oral/periodontal microbiota of different age groups

Reference	Number of subjects	Age groups (years old)	Microbiological technique	Sample	Summary of main results
Drake et al. (20)	Test 1: 375 PH (black) Test 2: 300 PH (white)	Mean Age: Test 1: 72.9 Test 2: 73.4	Immunofluorescence and BANA test *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia*	Subgingival biofilm	Older black adults had higher proportions of *Porphyromonas gingivalis, Prevotella* intermedia and BANA scores in the subgingival plaque than older white adults (p<0.05).
Marsh et al. (59)	120 PH (41 with partial dentures)	Four categories: 20–39 40–59 60–79 80	Microbial culture selective and non-selective media.	Supragingival biofilm and saliva	The proportions, mean log10 viable counts and the frequency of detection of yeasts and lactobacilli in saliva and plaque were consistently higher in partial-denture wearers (p<0.05). The proportions of *Staphylococcus* species and *Streptococcus mutans* were elevated in denture wearers, but these differences were not statistically significant.
Newman et al. (68)	7 PH	Mean: 63.0	Microbial culture selective and non-selective media.	Subgingival biofilm	Gram-positive organisms accounted for 50% of the predominant cultivable microbiota. *Streptococcus* species were the most frequently isolated organisms and were found in 83.8% of the samples.
Percival et al. (72)	79 PH	Four categories: 20–39 40–59 60–79 80	Microbial culture selective and non-selective media.	Supragingival biofilm and saliva	The total counts of viable bacteria in saliva, and the prevalence of *Streptococcus mutans* in supragingival plaque and saliva did not differ among age groups (p>0.05). Mean proportions, mean viable counts and the isolation frequency of *Lactobacillus* species were higher in the saliva samples of subjects 70 years old compared to those 39 years old (p<0.05). *Actinomyces naeslundii* was found in higher proportions in the younger age groups (20–39 and 40–59 years old), when compared with the older age groups and *Actinomyces oralis* was found in higher proportions in the oldest age groups 60–79, 80 years old (p<0.05).
Preza et al. (75)	Control: 20 PH Test: 21 PH with root caries	Mean: Control: 84.3 Test: 88.7	16S rRNA-based microarray for 300 species	Supragingival biofilm	*Lactobacillus casei/paracasei/rhamnosus* and *Pseudoramibacter alactolyticus* were associated with root caries biofilm samples. *Actinomyces* species were found more frequently in subjects from the control group (p<0.05).
Rodenburg et al. (78)	Test 1: 38 (severe periodontites) Test 2: 104 (refractory periodontites)	Test 1 and Test 2 groups presented the following age categories: 14–20 21–10 31–40	Microbial culture *Porphyromonas gingivalis, Prevotella intermedia* and *Aggregatibacter actinomycetemcomitans*	Subgingival biofilm	In Test 1 group the prevalence of *Aggregatibacter* actinomyceterncomitans-positive subjects and the number of those solely infected with *A. Actinomycetemcomitans* decreased with increasing age (p<0.05), whereas the prevalence of *P. gingivalis*-infected subjects increased with

Reference	Number of subjects	Age groups (years old)	Microbiological technique	Sample	Summary of main results
Savitt et al. (82)	1,492	Test 1 and Test 2 groups presented the following age categories: 10–19 20–29 30–39 40–49 50–59 60–69 41–70	DNA probes *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*	Subgingival biofilm	*Aggregatibacter actinomycetemcomitans* was correlated with subjects in the youngest age group (10 to 19 years old) with decreasing prevalence and levels in older age groups in comparison with the younger groups (p<0.05). In contrast, *Porphyromonas gingivalis* showed an inverse association with subject age, showing highest prevalence and levels in older groups (>30 years old) compared to subjects in younger age groups (10–29, 20–29 years old) (p<0.05). increasing age (p<0.05). However, these trends were not observed in the refractory periodontitis patients
Slots et al. (86)	1624 periodontitis subjects	Range: 15 to 89 Six categories: 15–24 25–34 35–44 45–54 55–64 >64	Microbial culture *Prevotella intermedia* and *Aggregatibacter actinomycetemcomitans*	Subgingival biofilm	*Aggregatibacter actinomycetemcomitans* was in higher prevalence (74%) in subjects <25years old than in adults and geriatric subjects (31%). *Prevotella intermedia* was present in 45% of the subjects and no predilection for a specific age group was detected.
Slots et al (87)	3075 "refractory" periodontitis	Range: 12 to 93 Fifteen categories: 10–14 15–19 20–24 25–29 30–34 35–39 40–44 45–49 50–54 55–59 60–64 65–69 70–74 75–79 >80	Microbial culture selective and non-selective media.	Subgingival biofilm	Older females (15.9%) and males (15.3%) had higher prevalence of enteric rods and pseudomonads than younger subjects (10.9%) (p<0.05). Older infected females yielded significantly higher viable counts of these microorganisms than younger infected females (p<0.05).

PH, periodontal health

Table 2

Demographic characteristics and mean (± SD) full-mouth probing depth (PD) and clinical attachment level (CAL) of subjects in each center

Variables	Experimental groups						
	Periodontitis			Periodontal health			
	Forsyth Institute n= 594	Guarulhos University n= 490	p-value	Forsyth Institute n= 193	Guarulhos University n= 53	p-value
Age (years)	49.6 ± 11.6	44.8 ± 12.0	0.000	37.8 ± 9.8	37.3 ± 15.8	0.756
Gender (% of Male)	54%	40%	0.126	37%	45%	0.091
Smokers (%)	29%	20%	0.056	12%	0%	0.000
PD baseline (mm)	3.4 ± 0.8	3.9 ± 0.7	0.000	2.5 ± 0.3	2.8 ± 0.1	0.000
CAL baseline (mm)	3.5 ± 1.1	4.4 ± 0.9	0.000	2.2 ± 0.7	2.5 ± 0.1	0.000

The significance of differences between the two databases was assessed using the Student T test. *SD, standard deviation*

Table 3

Demographic characteristics and mean (± SD) full-mouth probing depth (PD) and clinical attachment level (CAL) of the three age groups

Variables	Periodontitis				Periodontal health			
	Age groups			p-value	Age groups			p-value
	<35 years (n=152)	35–64 years (n=833)	>64 years (n=99)		<35 years (n=119)	35–64 years (n=112)	>64 years (n=15)	
Age (years)	28.5 ± 4.7 [A]	48.2 ± 7.6 [B]	69.6 ± 4.2 [C]	0.000	29.24 ± 6.2	44.1 ± 6.7	67.8 ± 9.8	0.000
Gender (% of Male)	47%	48%	49%	0.944	26%	32%	25%	0.630
Smokers (%)	16% [A]	21% [B]	19% [B]	0.013	26%	32%	25%	0.632
PD baseline (mm)	3.7 ± 0.8	3.6 ± 0.8	3.7 ± 0.8	0.080	2.6 ± 0.3	2.6 ± 0.3	2.7 ± 0.4	0.356
CAL baseline (mm)	3.8 ± 1.0 [A]	3.9 ± 1.1 [A]	4.2 ± 1.0 [B]	0.031	2.2 ± 0.6 [A]	2.3 ± 0.6 [A]	2.9 ± 0.2 [B]	0.000
% subjects from UnG	55%	43%	42%	0.054	26%	13%	50%	0.000

The significance of differences among age groups was assessed using the ANCOVA adjusted for smoking, gender, baseline PD and geographic population. The significance of differences between pairs of comparisons was determined using Tukey's multiple comparison test and are represented by different capital letters. *SD, standard deviation, UnG, Guarulhos University.*