Comparison of periodontal status between middle-aged human and the *Macaca fascicularis*

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Research

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Abstract

Background: Having an adverse impact on systemic health, periodontitis is an inflammatory disease which is characterized by the loss of integrity in tooth-supporting tissues. Middle-aged human (MAH) have been reported to have a higher incidence of periodontitis and a greater extent of bone loss. Although the similar dental anatomy and physiology as humans, whether middle-aged monkey (MAM) has the same disease phenotype as MAH is unclear. Furthermore, literature comparing the periodontal status between MAH and MAM is limited.

Methods: Therefore, four female middle-aged *Macaca fascicularis* and five middle-aged woman were used to evaluate the periodontal status before evaluating possible mechanism that may explain the difference between these two primates. CBCT examination and histological analysis were performed to characterize the periodontal condition, while 16s rRNA sequencing was done to explore the oral microbial profiles of two groups.

Results: The present study revealed a more severe disease phenotype in MAH than in MAM, along with significantly higher levels of alveolar bone loss and increased blood vessels and inflammatory cells infiltration. High-throughput gene sequencing demonstrated that the diversity of salivary microorganisms in MAH was lower than that of MAM. In addition, at the phylum level, the relative abundance of Proteobacteria in the MAH was higher than MAM, while Bacteroidetes showed a totally opposite trend. At the genus level, the relative abundances of *Alloprevotell*, *Aggregatibacter*, *Haemophilus*, *Gemella* and *Porphyromonas* in the MAH group were significantly less than that of the MAM group.

Conclusions: Altogether, these results demonstrated a potential link that may explain for the difference in inflammatory status in the oral cavity of MAH and MAM – the oral microbiota, prompting further investigation to explore specific roles of these bacterial population in the maintenance of oral health.

Background

Periodontal disease often presents as chronic infections in the oral cavity of adults, which can be characterized by the loss of supporting structures around affected teeth. Even though those who are 40 years old above are at higher risk to have periodontitis, a study in US showed that patients could develop periodontitis as early as 30 years old. The same study have also reported that some of them (7.8%) displayed symptoms fitting of severe periodontitis [1–5]. Apart from inflammation, one of the key features of severe periodontitis would be the presentation of alveolar bone loss [6]. Even though age is known to be a major risk factor, clinicians have discovered that patients could also display alveolar bone loss of 3 mm as early as 40 years old, with some of them ultimately suffer tooth loss given that there is not any supporting structures around the affected teeth [7, 8]. Zhao et al. investigated the prevalence and severity of alveolar bone loss in middle-aged (40–59 years) Chinese [2]. The results found that there was a higher degree of alveolar bone loss in females than males. Also, the incidence of bone loss in females was significant different from males. Furthermore, recent evidence has shown that periodontitis is

associated with the development of human chronic diseases in middle-age adults (e.g. type 2 diabetes, atherosclerosis, rheumatoid arthritis etc.) [9–11]. Thus, periodontal disease in the middle-aged population is attracting more attention and researchers are now trying to understand and tackle the disease of midl from multiple angles, especially the importance of oral microbiome.

Recognized as one of the most complex and dynamic microbial communities in the human body, the oral cavity consists of millions of microbes. Oral flora dysbiosis contributes to the enhanced development of periodontitis by interfering with the normal function of the host immune system [13]. In periodontitis, pathobionts and keystone pathogens such as *Porphyromonas gingivalis* appear in greater proportion than in health. The ‘red complex’, which appears later in biofilm development, comprises three species that are considered the most associated with disease: *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia* (Socransky et al., 1998; Holt and Ebersole, 2005). Contemporary sequencing technology revealed that many of these newly recognized organisms correlate with disease as strongly as the classical red complex bacteria, such as genera *Erysipelothrix*, *Solobacterium* and *Bulleidia* [14]. Given that oral microbial population changes as an individual ages, periodontitis can be observed with altered bacterial population along with inflammation in the oral cavity [15]. Xu et al. reported that salivary *Spirochaetes* abundance peaked in adults and declined in elders may partially explain why the Chinese population is more susceptible to periodontal diseases at adulthood compared with other age groups [16]. Furthermore, the relative abundances of predominant core bacteria in saliva are shown to change in the aging process [17].

Experimental models for periodontal diseases are essential for understanding the origin and evolution of the pathology in humans. As non-human primates represent animal models that most similar to humans in terms of dental anatomy and physiology, they also display periodontal diseases with clinical symptoms and host immune status that are comparable to humans [18–20]. To our knowledge, comparison of the periodontal status and oral microbiota of MAH and MAM have never been studied in-depth. Thus, the purpose of the present study was to characterize periodontal state of MAH and MAM before probing their oral microbiome to elucidate potential mechanisms that could explain for difference in inflammation status of the oral cavity.

Materials And Methods

Clinical samples

Patients (n = 5, female) included in this study were from 40 to 55 years of age. The patients had no uncontrolled medical conditions, generally normal blood chemistries, no need for corticosteroids or regular antibiotics during the last 3 months, were not pregnant, and were not taking drugs that would significantly affect the immune system (e.g., glucocorticoids or immunosuppressants). Oral clinical features included mild to moderate gingivitis, no probing depths (PD) > 3 mm, no history of smoking and periodontal disease, meeting these criteria. The tissue samples from the middle-aged woman were

collected from a corresponding site where tooth extractions for orthodontics or surgical removal of third molars was conducted.

Animal samples

For this study, four female *Macaca fascicularis* (11–15 years old, 2.6–3.6 kg) have been included. Each animal was offered a measured amount of a customized pellet feed. Fresh, potable drinking water was available to the animals *ad libitum*. The manner of CBCT and gingival tissue biopsies were performed similar to a human clinical study.

Acquisition of CBCT images

CBCT scans were performed with NewTom VG (Italy, Newtom) from Affiliated Stomatological Hospital of Guangzhou Medical University under the following conditions: 110 kV, 5 mA, 3.6 s exposure time. The scanning time was 24 s. The field of view measuring 100 mm in width and 100 mm in height. The original scanned data were analyzed through the image analysis module of the QR-NNT version 2.17 software. The percentage of attachment loss from the cemento-enamel junction (CEJ) to the root apex were defined as the assessment of the bone height changes. The percentage of attachment loss(%) = Attachment loss (b) (mm)/A length of CEJ to root apex (a) (mm). The attachment loss were measured at six sites ranging from the disto-palatal or disto-buccal groove of the second premolar to the second molar palatal or buccal cusp. The data measurement were carried out by the same observer on two separate occasions within a 4-week interval.

Histological analysis

Gingival tissues of MAH and MAM were detected by HE and immunohistochemistry with decalcified paraffin embedded sections at 4 µm thickness. Histologic images were captured (DMLS; Leica) and analysed by Image-J software (NIH, Bethesda, MD, USA). For HE staining, the number of inflammatory cells and MVD in four unit squares (50 µm × 50 µm) of periodontal connective tissue was counted at an objective magnification of 40 × and then averaged. For IHC staining, Primary anti-CD34 (Boster, China) and anti-CD45 (Boster, China) was used and detected by secondary Anti-rabbit IgG (ZASGB, China) followed by DAB substrate staining (PerkinElmer, Waltham, Massachusetts). The quantitative analysis of the number of positive cells was determined by counting the number of stained cells in the resorption areas under a microscope. At least 4 samples in each group were used for analysis.

Characterization of the oral microbiome using 16 s rRNA genes

Unstimulated human (n = 5) and animal saliva samples (n = 4) were collected between 8:00 a.m. and 11:00 a.m. according to manufacturer's instructions (Salivette, Germany). Immediately after collection each saliva sample was centrifuged for two minutes at 1000 rpm. Then the samples were immediately stored at 4 °C until further processing. genomic DNA of each sample was extracted using the Fecal genomic DNA extraction kit(Tiangen biotech CO., LTD, China) according to the manufacturer's preparation protocol within 24 hours after collection.

The quantity and quality of extracted DNA were measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and agarose gel electrophoresis, respectively. The results showed that the A260/A280 ratios were all between 1.8 and 2.0 and that the DNA concentrations were 20–150 ng/IL, indicating that the extracted genomic DNA was ideal and met the requirements for subsequent sequencing.

Extracted DNA (20 ng) from each sample was PCR amplified according to the TruSeq Nano DNA LT Library Prep Kit for library preparation. The 16S rDNA V4 area specific primer 520F(5'-barcode + AYTGGGYDTAAAGNG-3'), 802R(5'-TACNVGGGTATCTAATCC-3') were used to amplify the V4 region of 16S ribosomal DNA. High throughput sequencing was performed by Shanghai Personal Biotechnology Co., Ltd with MiSeq Reagent Kit V3 (600cycles) paired-end runs. A total of 3,138,666 reads were generated with a mean of $39,098 \pm 4421$ reads per sample. Sequences with $\geq 97\%$ similarity were assigned to the same operational taxonomic units (OTUs) by Vsearch (v2.3.4). Representative sequences were chosen for each OTU, and taxonomic data were then assigned to each representative sequence using the RDP (Ribosomal Database Project) classifier. The differences of the dominant species in different groups, multiple sequence alignment were conducted using the mafft software (V 7.310) to study phylogenetic relationship of different OTUs. OTUs abundance information were normalized using a standard of sequence number corresponding to the sample with the least sequences. Alpha diversity is applied in analyzing complexity of species diversity for a sample through 5 indices, including Chao1, Observed species, Goods_coverage, Shannon, Simpson and All this indices in our samples were calculated with QIIME (Version 1.8.0). Beta diversity analysis was used to evaluate differences of samples in species complexity. Beta diversity were calculated by PCoA and cluster analysis by QIIME software (Version 1.8.0).

Statistical Analysis

Statistical analysis of microbial samples is given in the Sequencing Data Analysis section above. Statistical analysis for all other experiments was performed using GraphPad Prism software from GraphPad Software Inc. (La Jolla, CA, USA). All data were expressed as the mean $\pm$ SEM. The difference between two groups was established by the Student's t-test. Multiple group comparisons were performed by one-way ANOVA, with Tukey's or Bonferroni's post-hoc test to identify differences between specific groups. A value of $P < 0.05$ was considered to be statistically significant. No analysis was performed to determine whether the data met assumptions of the statistical approach. Each sample examined was from a different animal and the individual animal was the unit of measurement. The number of animals examined per group and the number of times the experiments were carried out are given in the Methods above and in the Figure Legends. For all experiments a minimum sample size of 5 was required based on our previous experience and published studies.

Results

Evaluation of bone volume of MAH and MAM using CBCT

A characteristic feature of periodontitis is loss of bone around the teeth. Periodontal bone loss typically reflects the degree of present inflammation. As shown in Fig. 1a, the alveolar bone around the posterior teeth in MAH was obviously resorbed. However, little bone resorption was seen surrounding the posterior teeth in MAM. Quantitative analysis of the CBCT results demonstrated that the percentage of posterior teeth's alveolar bone loss in MAH were $20.3 \pm 2.8\%$, $28.8 \pm 5.2\%$, and $22.3 \pm 4.6\%$, respectively. For MAM, the corresponding results were $6.7 \pm 1.6\%$, $13.8 \pm 3\%$, and $10.1 \pm 2\%$. It is very evident that the percentage of attachment loss around posterior teeth were all significantly higher in middle-aged woman than in middle-aged *Macaca fascicularis* ($P < 0.05$, Fig. 1b). This result indicated that the average reduction in maxillary posterior alveolar bone height of MAH were more severe than that of MAM.

Inflammation level of gingival tissues from MAH and MAM via HE and IHC

For more information of gingival tissue about MAH and MAM, histological analysis were performed. When analyzing the MAH group, histological aspects of gingival samples showed the enlargement of spinocellular layer, acanthocyte edema, epithelial atrophy and rich inflammatory cells infiltrate in connective tissue. However, when analyzing microscopic aspects of gingival sections of the MAM group, histological examination of gingival samples illustrated healthy aspects of epithelium and connective tissue. In addition, histological study indicated an increasing number of inflammatory cells and blood vessels in MAH group gingival mucosal lamina propria, while no obvious histopathological changes were found in MAM gingival tissue (Fig. 2).

To understand whether the severe periodontal tissues inflammation in MAH was due to the promotion of angiogenesis and cell proliferation, expression of CD34 and CD45 in gingival tissues were analyzed. As we predicted, the number of CD34-positive and CD45-positive cells in MAH were significantly higher than in MAM group (Fig. 3a). Immunohistochemical staining revealed more neovasculature in the tissues from MAH compared with MAM (Fig. 3a). Statistical analysis further indicated dramatically increases of MVD and inflammatory cells in the tissues of MAH when compared with the MVD in the tissues of MAM (Fig. 3b). These results revealed that inflammation state of gingival tissues surrounding the dentition was more severe in the MAH group than in the MAM group. In summary, more severe periodontal disease phenotype was found in MAH than in MAM, along with significantly higher levels of alveolar bone loss and increased microvasculature and inflammation cells infiltration.

Differences of salivary microbiome of MAH and MAM with 16 s rRNA

Six known phyla were represented among the total OTUs. In the human oral samples, five phyla had relative abundances greater than 1%: Proteobacteria (67.6%), Firmicutes (31.9%), Bacteroidetes (1.98%), Actinobacteria (3.7%), and Fusobacteria (1.2%). In the MAM oral samples, five phyla had relative abundances greater than 1%: Firmicutes (34.1%), Proteobacteria (32.3%), Bacteroidetes (18.1%), Actinobacteria (5.3%), and Fusobacteria (9.6%) (Fig. 4). Both the MAH and MAM salivary microbiome were dominated by Firmicutes, followed by Proteobacteria. At the phylum level, the most striking difference was the much higher abundance of Proteobacteria in the MAH group, while the Bacteroidetes in the MAH group showed a reverse trend ($P < 0.05$).

In the human oral samples, the bacterial taxa with > 1% abundance were *Streptococcus* (26.5%), *Neisseria* (26.8%), *Acinetobacter* (11.4%), *Rothia* (5.2%), *Granulicatella* (3.2%), *Yesinia* (2.68%), *Porphyromonas* (1.7%), *Gemella* (1.6%), *Prevotella* (1.1%). In the MAM oral samples, the bacterial taxa with > 1% abundance were *Streptococcus* (21.1%), *Porphyromonas* (10.1%), *Neisseria* (8.3%), *Fusobacterium* (7.7%), *Granulicatella* (7.6%), *Gemella* (6.1%), *Capnocytophaga* (4.3%), *Haemophilus* (4.2%), *Alloprevotella* (2.7%), *Aggregatibacter* (2.6%), *Leptotrichia* (2.1%), *Bacillus* (1.1%) (Fig. 4). Moreover, *Neisseria* was much more dominant in MAH than in MAM. Besides, the relative abundances of *Alloprevotella*, *Aggregatibacter*, *Haemophilus*, *Gemella* and *Porphyromonas* in the MAH group were less than that of the MAM group obviously, while the TM7 showed the opposite result, the difference was statistically significant.

The diversity, evenness, and means of each value in MAH and MAM are presented in Fig. 5. This result indicated that observed species richness (Fig. 5a), Chao-1 index (Fig. 5b), and Shannon diversity index (Fig. 5c) of MAM oral microbiome were richer than that of MAH, and the difference was statistically significant ($P < 0.05$). The results of alpha diversity indices for MAH and MAM samples revealed that the MAM oral microbiota was more diverse than that in MAH group. It is accepted that this more diverse community represents a more stable and healthy ecosystem. This diversity may result from subverting defenses that would limit community composition to non-pathogenic commensals.

Difference between the salivary microbial profiles of MAH and MAM was evident as determined by principal coordinates analysis (Fig. 6). UniFrac analysis showed a certain difference in the community structure between samples. Furthermore, the MAH and MAM samples tended to cluster separately and the MAH samples indicated lower intra-sample variability in comparison with the MAM samples.

Discussion

To our knowledge, the comparison of periodontal status between human and non-human primates have not been reported previously. In the present study, we characterized the periodontal status by CBCT and histological methods and the primary finding was that more severe periodontal disease phenotype was examined in MAH than that in MAM. As reported, the prevalence and severity of marginal inflammation increase with age. A similar observation was also seen in cynomolgus monkeys as inflammation in the oral cavity correlates positively with age and duration of captivity [21, 22]. However, we surprisingly found that the alveolar bone of MAM were in a healthy state without significant resorption. This result is contradicted by other studies suggesting obvious alveolar bone loss in middle-aged non-human primates [11, 18, 21], which should arouse our high attention. Also, our results showed that the amount of alveolar bone resorbed in MAM was significantly less than MAH. A previous report found a greater degree of bone loss in middle-aged woman when comparing with the current study [2]. The possible reason was that the subjects selected were menopausal or/and smokers. As we expected, the results from histological analysis of gingival tissue showed higher number of inflammatory cells in MAH compared with that of MAM, which further confirmed the radiography result.

As periodontitis is thought to be a chronic inflammation of local tissues surrounding the teeth, the differences in periodontal inflammation between middle-aged and elderly human could be related to microbial composition and diversity [21, 23, 24]. As an attempt to search for possible underlying mechanisms behind the above interesting results, oral saliva samples were randomly collected from middle-aged human and monkeys, and analysis on the microbial profile was done based on 16 s RNA gene sequences.

It has been suggested that the host and the microbe are in a co-evolutionary relationship. Compared with human, the cynomolgus monkey has a similar genetic composition, oral structure, and oral disease, which makes the oral microbes similar in composition. However, the oral cavity microorganisms are affected by many internal and external factors, which make oral microbes have difference in richness. This study yielded similar experimental results. At the level of phylum, MAH shows a higher abundance of Proteobacteria and a lower abundance of Bacteroidetes compared to the MAM group, differential oxygen levels might be one driving physical factor shaping the oral habitats represented by the salivary microbiome in humans and monkeys, similar result was reported in Philippot's study [25]. Griffen et al. reported similar results as the present study by comparing the periodontally healthy controls and subjects with chronic periodontitis, where Bacteroidetes were found health-associated, whereas Proteobacteria and Erysipelotrichia were associated with disease [26, 33].

For the genus level, results showed that MAH showed higher abundance of TM7 and lower abundance of Aggregatibacter, Haemophilus, Gemella and Porphyromonas. Surprisingly, neither Aggregatibacter, Haemophilus, Gemella, Fusobacterium, nor Porphyromonas and Haemophilus were found to be significantly more abundant in the MAH disease samples, despite being previously implicated in this disease [27–30]. This is likely due to the high variance in the abundance of these organisms across our samples, as well as the small sample size which affects our statistical power. TM7 is a novel candidate bacterial division with no cultivated representatives, and previous studies have shown microbes from this division to be commonly found in the human oral flora but at relatively low abundance, generally around 1% of the population, which almost the same as the present study. Despite of that, the TM7 division was statistically enriched in MAH samples. Liu's study indicated that this division had correlation with periodontal disease, but its role in disease have yet to be fully appreciated [31]. Also, other researchers suggested that the presence of a few uncultivable species such as TM7, could be highly significant for the manifestation of oral diseases, particularly periodontal disease [31–33]. Moreover, we observed high numbers of members of the genus Neisseria in the MAH samples, which in accordance with the periodontal diseased samples in Frias-Lopez's report [34, 35]. Above all, we speculated that Proteobacteria, TM7, and Neisseria might have closer relationship with the periodontal tissue destruction, the potential mechanism was that the pathogens encoded chitinases that support infection of non-chitinous mammalian hosts, possibly by suppressing host innate immunity [34].

A more diverse bacterial community is considered to represents a more stable and healthy ecosystem [26]. In fact, oral diseases have been associated with a decrease in bacterial diversity. In accordance with previous authors [26, 36–38], the present study indicated that MAM group had higher alpha diversity than

the MAH group. The higher oral microbial diversity of elderly monkeys in this study may be related to their feeding environment and eating habits. Also, in comparison with MAH, a number of low redundancy oral microbiotas were existed on the MAM, which may be related to the feeding mode. As such they might represent only transient species, given that the indigenous microflora is usually able to defend its ecological niches successfully against foreign bacteria [39]. This barrier against foreign bacteria is based on interactions between the indigenous microflora and the immune system, which in turn is the result of long-term coevolution in animals [37]. However, the interplay between the immune system and indigenous microflora might work best in the natural habitat, where it evolved. The conspicuous high number of low-abundance bacteria in MAM might indicate that this balance is partially disrupted and that eventually at least some of the novel bacteria may be able to occupy distinct oral niches.

Although non-human primates have the similar oral microbial composition with human, differences in microorganisms between these species were existed [40]. Internal factors, related to phylogeny or host physiology, have a more important influence on the saliva microbiome than geography or local environment [37]. Moreover, a recent study of mothers and infants found a higher correlation among the microbiomes of infants and of mothers than of infants with their mothers [41], suggesting that diet related aspects of host physiology may play a role. Other reports demonstrated that a new human-specific diet have resulted in obvious changes to alterations in the oral microbiome [42–44]. Diet composition of MAM and MAH were significantly different (Table S1). Our results are compatible with dietary factors related to host physiology (e.g., proportion of meat in the diet) as the primary influences on the saliva microbiome. In line with our finding, another report compared the proteomes of human saliva and the saliva of our closest extant evolutionary relatives, chimpanzees and gorillas and opened up the possibility that dietary differences may have shaped a distinct salivary proteome in the human lineage [41]. Therefore, dietary factor may shape salivary microbial profiles, which might contribute to the alternations of periodontal status.

Conclusions

The current study first compared the naturally periodontal status of MAH and MAM. Unexpectedly, periodontal tissues of MAM was found in a healthy state and more severe periodontal tissues inflammation level was detected in the MAH group. The possible reason were that dietary factors may have influence on the oral microbial composition and diversity, which in turn caused the change of periodontal status. These findings suggest that possible novel diet-shaped oral microbiota could provide some guidance regarding the apparent “resistance” of the periodontal tissue in MAM eliciting an inflammatory response. However, we still have little understanding of how dietary factors influenced the oral microbiome and the mechanisms of the oral microbiome contributes to the development of periodontal tissue destruction. Knowledge of this process will potentially help to clarify the dietary habitats that could translate into longer-term risk for periodontal disease, as well as focusing efforts on approaches to effectively modulate the microbial acquisition by individuals to improve long term oral health.

Abbreviations

MAH, middle-aged human; MAM, middle-aged *Macaca fascicularis*; CBCT, cone beam computed tomography; US, United States; MVD, microvessel density; CEJ, cemento-enamel junction; HE, hematoxylin-eosin staining; IHC, Immunohistochemistry; OTU, Operational taxonomic unit; PCoA, principal coordination analysis;

Declarations

Ethics approval and consent to participate

All experiments were approved by the Guangdong Laboratory Animals Monitoring Institute (IACUC201806). All human participants signed the informed consent before CBCT examination and collecting the samples. Each of the human studies was reviewed and approved by the Institution Ethics Committee of Stomatology Hospital of Guangzhou Medical University.

Consent for publication

Not applicable.

Availability of data and material

The datasets supporting the results of this article are included within the article (and its additional files).

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

LBP, ZM and GHB helped in the sample collection, gathering of participant data, writing original draft. SHL and LHH helped in the manuscript writing, data analysis, and interpretation. ZZJ helped in the HE and IHC assays, and final approval of manuscript. WLH and CJM helped in the research design, analysis and interpretation of results. WLJ and GLH conceived of the study, and participated in its design and coordination. All authors read and approved the final manuscript.

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Authors' information

Not applicable

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Supplementary Table

Supplementary Table 1 is not provided with this version of the manuscript.

Figures

Figure 1

Relative alveolar bone loss of maxillary posterior teeth between MAH and MAM. (a) The alveolar bone around the posterior teeth in MAH was obviously resorbed. (b) Little bone resorption was seen surrounding the posterior teeth in MAM. Scale bar: 5 mm. *: $P < 0.05$, **: $P < 0.01$.

Figure 2

Histological characteristics of gingival tissues in MAH and MAM samples. The enlargement of spinocellular layer, acanthocyte edema, epithelial atrophy and rich inflammatory cells infiltrate were

found in connective tissue of MAH samples. While histological examination of gingival samples in MAM illustrated healthy aspects of epithelium and connective tissue. Scale bar: 100 μ m.

Figure 3

Expression of CD 34 and CD 45 in gingival tissues of MAH and MAM. (a) The number of CD34-positive and CD45-positive cells in MAH were significantly higher than in MAM group. (b) The statistical analysis demonstrated that expression of both CD34 and CD45 were higher in the tissues from MAH than that in the tissues of MAM. Scale bar: 50 μ m. ***: $P < 0.001$.

Figure 4

Relative distribution of sequences in the OTUs of the 9 oral samples at at the phylum level and genus level. Stacked bar graphs illustrate the abundances of phyla and genus. In the human oral samples, five

phyla had relative abundances greater than 1%: Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, and Fusobacteria. In the MAM oral samples, five phyla had relative abundances greater than 1%: Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria, and Fusobacteria. At the genus level, *Neisseria* was much more dominant in MAH than in MAM. Besides, the relative abundances of *Alloprevotella*, *Aggregatibacter*, *Haemophilus*, *Gemella* and *Porphyromonas* in the MAH group were less than that of the MAM group obviously.

Figure 5

Alpha diversity index of MAH and MAM. Measures of diversity, (a) observed species richness, (b) chao-1 index, and (c) shannon diversity index were all lower in MAH than in MAM. (* p < 0.05, ** p < 0.01)

Figure 6

Microbiota separation on the principal coordinates calculated from unweighted UniFrac distances. The MAH and MAM samples tended to cluster separately and the MAH samples indicated lower intra-sample variability in comparison with the MAM samples. (Red dots indicate MAH, while blue dots indicate MAM.)