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

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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 middle-aged monkey (MAM) shares similar dental anatomy and physiology with humans, it is still unclear whether they both display same disease phenotype or not, given that literature comparing the periodontal status between MAH and MAM is limited. CBCT examination and histological analysis were performed to characterize the periodontal condition, while 16 s rRNA sequencing was carried out to evaluate the possible mechanism that may explain the difference between these two primates.

Results

The present study revealed a more severe disease phenotype in MAH than in MAM, along with significantly higher levels of alveolar bone loss accompanied with increased blood vessels and inflammatory cells infiltration. High-throughput gene sequencing based on 16S rRNA gene 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. One of the possible factor that may explains for this observation would be the dietary factors, affecting the oral microbial composition and diversity, and subsequently contributing to the alterations of periodontal status.

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 normal health. The ‘red complex’, which appears later in biofilm development, comprises of three species that are considered the most associated with disease: P. 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 age, periodontitis can be observed with altered bacterial population along with inflammation in the oral cavity [1, 15–16]. 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 [17]. Furthermore, the relative abundances of predominant bacteria in saliva are shown to change in the aging process [18].

Experimental models for periodontal diseases are essential for the understanding the origin and evolution of the pathology in humans. As non-human primates represent animal models that closely resemble 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 [19–21]. Nevertheless, 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.

Results

Evaluation of bone volume of MAH and MAM using CBCT

A prominent feature of periodontitis is characterized by the loss of bone around teeth which typically reflects the degree of present inflammation. As shown in Fig. 1a, the alveolar bone around the posterior teeth in MAH was obviously resorbed. On the contrary, 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 ranged from 20.3 (± 2.8%) to 28.8 (± 5.2%), while the results for MAM were shown lower value ranged from 6.7 (± 1.6%) to 13.8 (± 3%). Thus, it is very evident that the percentage of attachment loss around posterior teeth was significantly higher in MAH than in MAM ($P < 0.05$, Fig. 1b). In addition to that, result also indicated that more severe reduction in maxillary posterior alveolar average bone height in MAH compared to that of MAM.

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

In order to visualize and compare the inflammatory changes within gingival tissue of MAH and MAM, histological analysis was performed. Firstly, the gingival sections of the MAH group displayed significantly altered structures: (a) epithelium appears acanthosis (enlargement of spinocellular layer) and atrophy and (b) infiltration of inflammatory cells 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. Besides, there is a marked change in lamina propria with higher number of inflammatory cells and blood vessels observed in MAH samples, but little of these present in the MAM gingival tissue (Fig. 2). As an attempt to determine whether the severe periodontal inflammation in MAH was caused by promotion of angiogenesis and/or cell proliferation, expression of CD34 and CD45 in gingival tissues were studied thoroughly. As anticipated, the number of CD34-positive and CD45-positive cells in MAH were significantly higher than in MAM group (Fig. 3a). In addition, immunohistochemical staining revealed more neovascular in the tissues from MAH compared with MAM (Fig. 3a), marked by a dramatically increase in MVD and number of lymphocyte detected (Fig. 3b). Overall, results from histological analysis emphasized that inflammation was more prominent and severe in the MAH group than in the MAM group, in addition to significantly advanced alveolar bone loss and increased blood vessles.

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

Six known phyla were represented among the total OTUs. Out of which, five phyla exhibited relative abundances greater than 1% in both salivary samples of MAH and MAM: *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Fusobacteria* (Fig. 4). In fact, both MAH and MAM salivary microbiome were predominantly members of *Firmicutes* and *Proteobacteria*, albeit with different abundance. There was a higher abundance of *Proteobacteria* than *Bacteroidetes* in the MAH group, while MAH group showed a reverse trend with *Bacteroidetes* ($P < 0.05$). At genus level, there were nine bacterial taxa with >

1% abundance in MAH salivary samples: *Streptococcus* (26.5%), *Neisseria* (26.8%), *Acinetobacter* (11.4%), *Rothia* (5.2%), *Granulicatella* (3.2%), *Yersinia* (2.68%), *Porphyromonas* (1.7%), *Gemella* (1.6%), *Prevotella* (1.1%), whereas twelve bacteria taxa showed > 1% abundance in MAM salivary samples with highest observed in *Streptococcus* (21.1%), followed by *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). Additionally, the differential profiles between MAH than in MAM could also be explained by abundance of genera like *Alloprevotella, Aggregatibacter* and *Haemophilus* which is significantly higher in MAM than MAH group, while *TM7* showed the opposite trend – significantly lower in MAM compared to MAH group.

The diversity, evenness, and means of each value in MAH and MAM are presented in Fig. 5. Based on the scores from observed species richness (Fig. 5a), Chao-1 index (Fig. 5b), and Shannon diversity index (Fig. 5c), the oral microbiome of MAM were richer than that of MAH ($P < 0.05$). On the other hand, 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 generally accepted that a 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 PCoA (Fig. 6). UniFrac analysis showed a major difference in the community structure between samples. Furthermore, the MAH and MAM samples formed two distinct separate cluster and the MAH samples indicated lower intra-sample variability in comparison with the MAM samples.

Discussion

The oral cavity is second to gut in terms of richness and diversity of microbiota, consists of over 700 species of prokaryotes [22]. In reality, the oral microbiome starts to form shortly after birth, invaded by species such as *Streptococcus, Lactobacillus, Actinomyces, Neisseria* and *Veillonella*. Besides protecting the oral cavity, the oral microbiome may play an important role in determining disease initiation and progression [23]. Generally speaking, a highly diverse bacterial community is portrayed as a more stable and healthy microbiome [33]. In fact, oral diseases have been associated with a decrease in bacterial diversity. Periodontitis thought to be a chronic inflammation of local tissues surrounding the teeth and occurrence and severity of marginal gingival inflammation tends to be higher in the older population around the world [24, 25]. Interestingly, similar observation was also seen in cynomolgus monkeys as inflammation in the oral cavity correlates positively with age and duration of captivity [26, 27]. It was then discovered that the differences in severity of inflammation between middle-aged and elderly human could be related to microbial composition and diversity in the oral microbiome [26, 28, 29]. Furthermore, to our knowledge, the comparison of periodontal status between human and non-human primates have not been reported previously. In the present study, we examined and compared the periodontal health of MAH

and MAM using imaging technique like CBCT in combination with histological methods and next generation sequencing to explore the role of oral microbiome in periodontitis.

Based on CBCT results, MAH displayed a more severe periodontal disease phenotype compared to MAM. Even though they were at middle-aged, the alveolar bone of MAM were surprisingly found to be in a healthy state, without significant resorption. In reality, MAH displayed significant bone loss which is also observed by another studies which described similar observation where age seems to be one of the major risk in developing periodontitis [2, 30]. One of the possible explanations is that the subjects selected were post-menopausal or/and smokers, these lifestyle factors could be confounding factors for development of periodontal diseases and account for the differences seen between MAH and MAM [31]. Nonetheless, results from histological analysis of gingival tissue also showed higher number of inflammatory cells in MAH compared with that of MAM, which complements with the data obtained from radiography investigations. 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.

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 a driving physical factor shaping the oral habitats represented by the salivary microbiome in humans and monkeys as reported by Philippot and team [32]. At the genus level, compared with MAM, results from MAH showed higher abundance of *TM7* and lower abundance of *Aggregatibacter*, *Haemophilus*, *Gemella* and *Porphyromonas*. Surprisingly, the relatively low abundance of *Porphyromonas, Fusobacterium, Aggregatibacter*, and *Haemophilus*, which considerd as the disease-associated species, were found in MAH samples, despite being previously implicated in the diseased individuals [33–36]. The possible reason are the high inter-individual variability in the abundance of these samples, as well as the small sample size [36]. *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 present within 1% of the population, which is highly similar to the present study. Despite of that, the *TM7* division was statistically enriched in MAH samples. A study by Liu et al. indicated that this division had some correlation with periodontal disease, but its role in disease have yet to be fully appreciated [37]. Also, other researchers suggested that the presence of a few uncultivable species such as *TM7*, could be highly important for the manifestation of oral diseases, particularly periodontal disease [37–39]. Additionally, we observed high numbers of *Neisseria* sp. in the MAH samples, which in accordance with the periodontal disease samples as reported previously [40, 41]. Above all, we speculated that *Proteobacteria*, *TM7*, and *Neisseria* might have a closer association with periodontal tissue destruction. A study from Netherlands mentioned that whole saliva of periodontitis patients contains higher level of chitinase than healthy controls. The increased level of chitinase in these patients dropped significantly after periodontal treatment for 5–6 months [42]. Even though chitinase can also be produced by mammals, but their levels are increasingly associated with inflammation and the production of chitinases by microbes such as TM7 can cause infection of non-chitinous mammalian hosts, via the suppression of host innate immunity [40, 42–44].

As non-human primates, cynomolgus monkeys shares a similar genetic composition, oral structure and oral disease with human. These findings, in turn leads to the assumption that these two groups would probably reflect a highly similar oral microbial profile [45, 46]. However, the oral cavity microorganisms are affected by many internal and external factors, including smoking, dietary intake, which then results in difference in oral microbial composition [48]. In accordance with previous authors [48–51], the present study indicated that MAM group had higher alpha diversity than the MAH group. Even so, it is worth to take note that non-human primates in samples may differ from those living in the wild [52]. As their diet did not consist of the full diversity of plants/foods these animals would normally eat in the wild, the microbiome may seem to be “humanized”, marked by the colonization of modern human microbiome [52–56]. The diet composition of human and non-human primate were significantly different [51]. Nevertheless, current results are consistent other studies looking how dietary factors presents as the primary influence on the salivary bacteria. Tian and his team indicated that carbohydrate-rich diet was responsible for the abundant aciduric and acidogenic salivary bacteria in human saliva [57]. Highly exposed sugar to teeth and starch consume might cause the growth of periodontitis along with enhanced bacteria in humans in comtrast to the great apes [58]. Therefore, dietary factor may shape salivary microbial profiles, which might contribute to the alternations of periodontal status.

Conclusions

The current study is one of the first which compares the naturally periodontal status of MAH and MAM. Interestingly, periodontal tissues of MAM was found to be healthier than MAH which exhibited extensive inflammation level. One of the possible factor that may explains for this observation would be the dietary factors, affecting the oral microbial composition and diversity, and subsequently contributing to the alterations 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, little understanding is available on 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.

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 no 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, body weight of 2.6–3.6 kg, Guangdong Chunsheng Biotechnology Co., Ltd) 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. Before sample preparation, subjects were anesthetized with of ketamine (15 mg/kg) administered intramuscularly and bled to death. The manner of CBCT and gingival tissue biopsies were performed similar to a human clinical study.

Acquisition of cone beam computed tomography (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 cementoenamel junction (CEJ) to the root apex were defined as the assessment of the bone height changes. The percentage of attachment loss(%) = An 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 H&E staining 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 microvessel density (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 lgG (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). After collection, saliva sample

was centrifuged for two minutes at 1000 rpm and 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. All samples were processed within 24 hours after collection.

The quality control 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 A_{260}/A_{280} ratios were all between 1.8 and 2.0 and that the DNA concentrations were 20–150 ng/μL, 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 ± 4421 reads per sample. Sequences with ≥ 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 ± 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.

Abbreviations

MAH, middle-aged human; MAM, middle-aged Macaca fascicularis; CBCT, cone beam computed tomography; US, United States; MVD, microvessel density; CEJ, cementoenamel 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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Figures

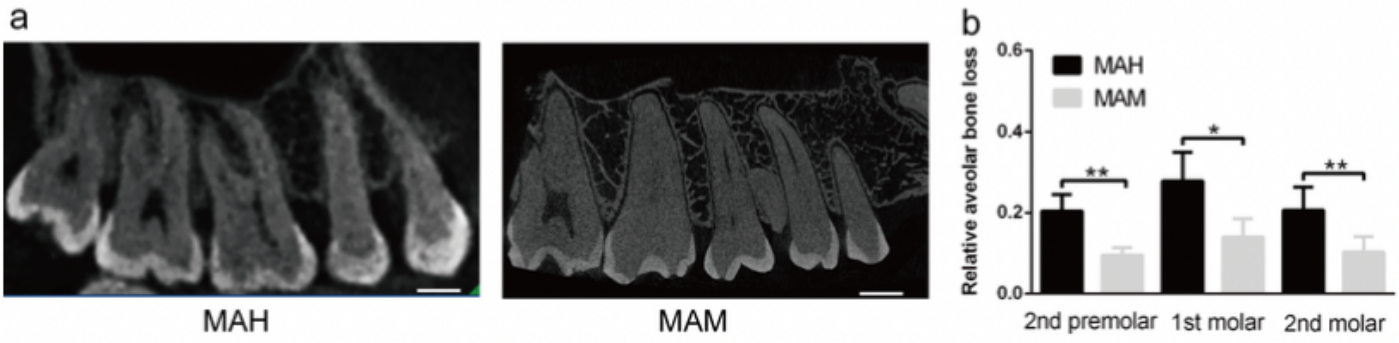

Figure 1

Relative aveolar bone loss of maxillary posterior teeth between MAH and MAM. (a) The aveolar 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. *: $P < 0.05$, **: $P < 0.01$.

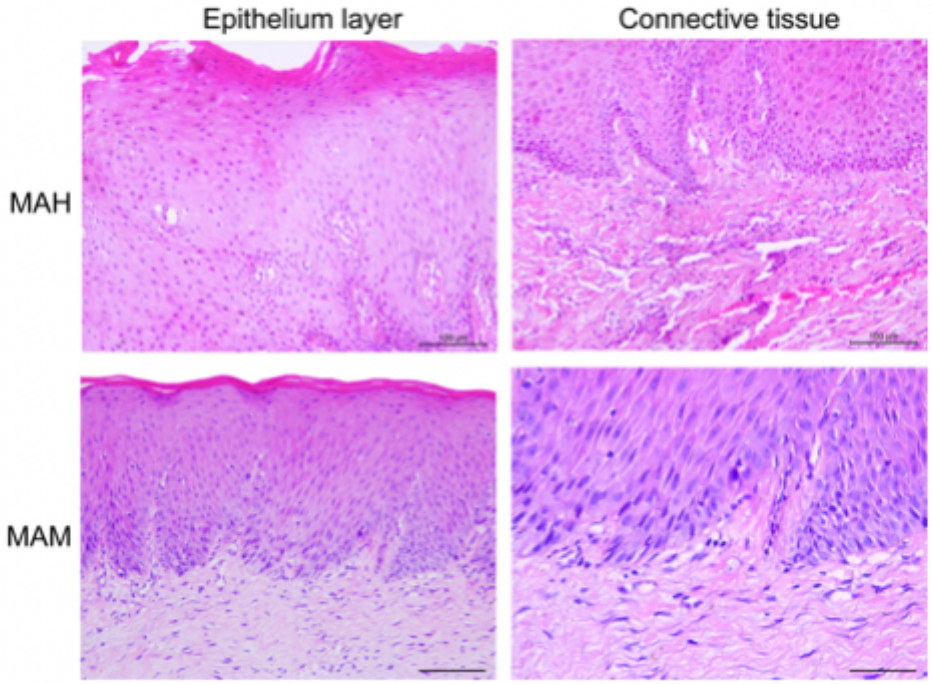

Figure 2

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 μm.

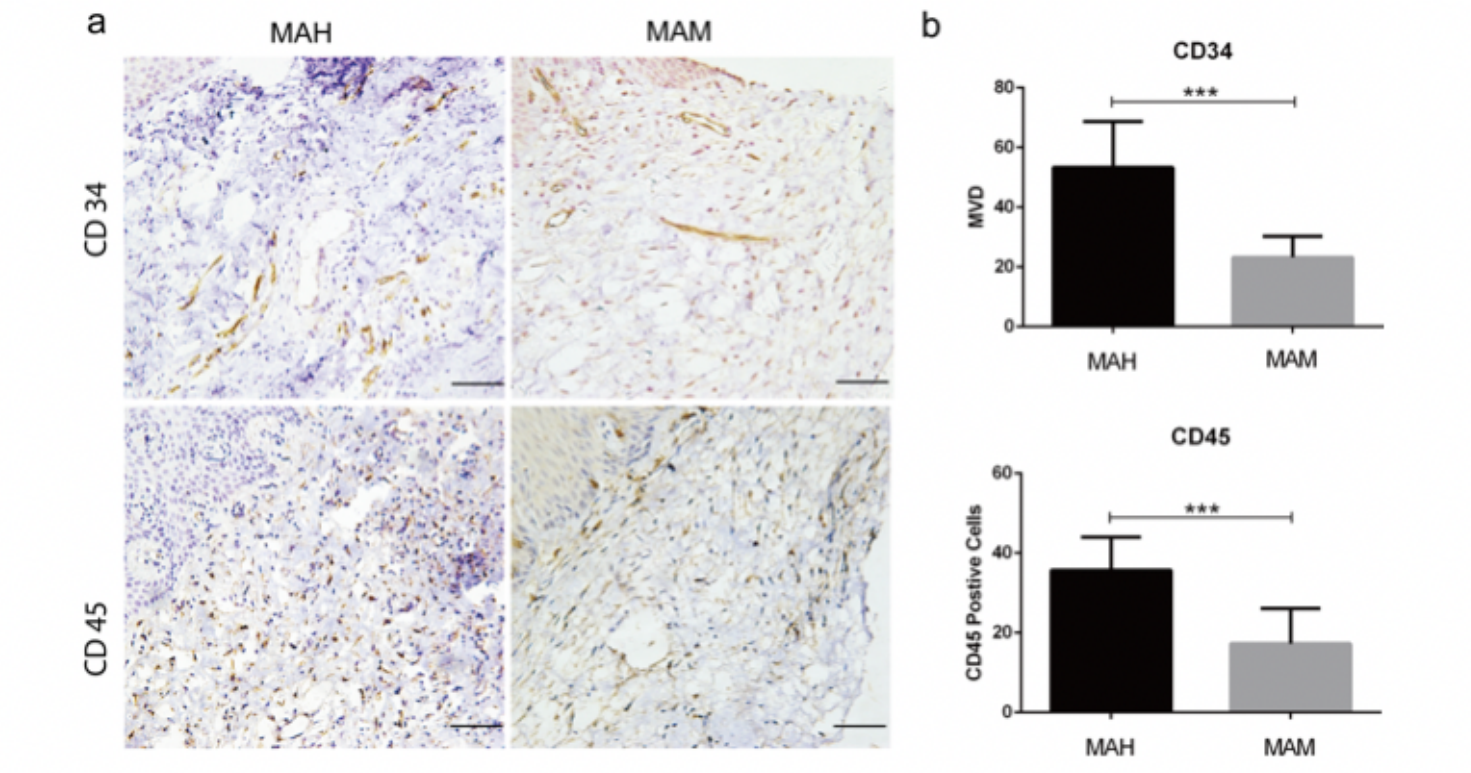

Figure 3

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 μm. ***: P < 0.001.

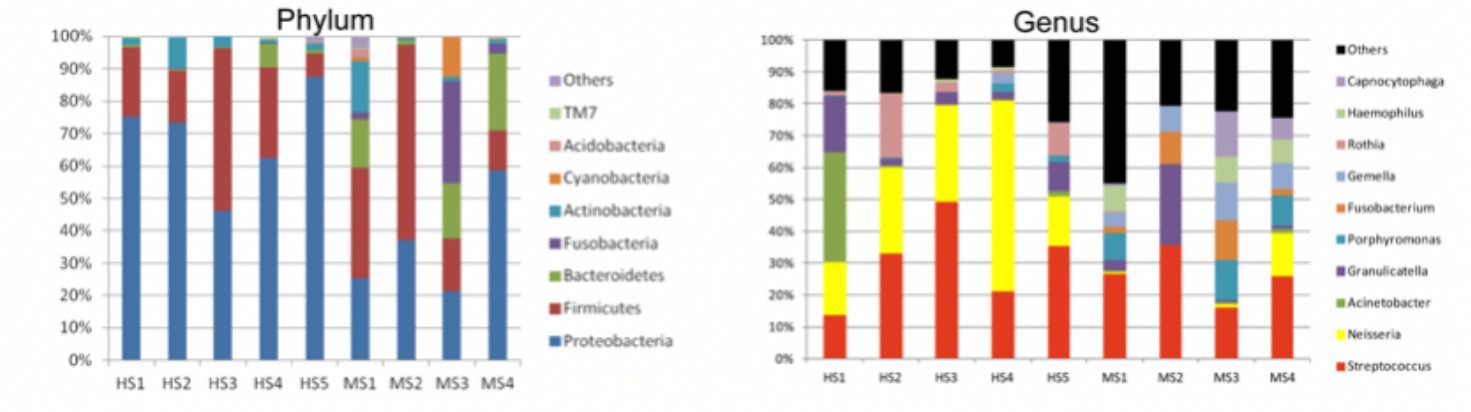

Figure 4

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.

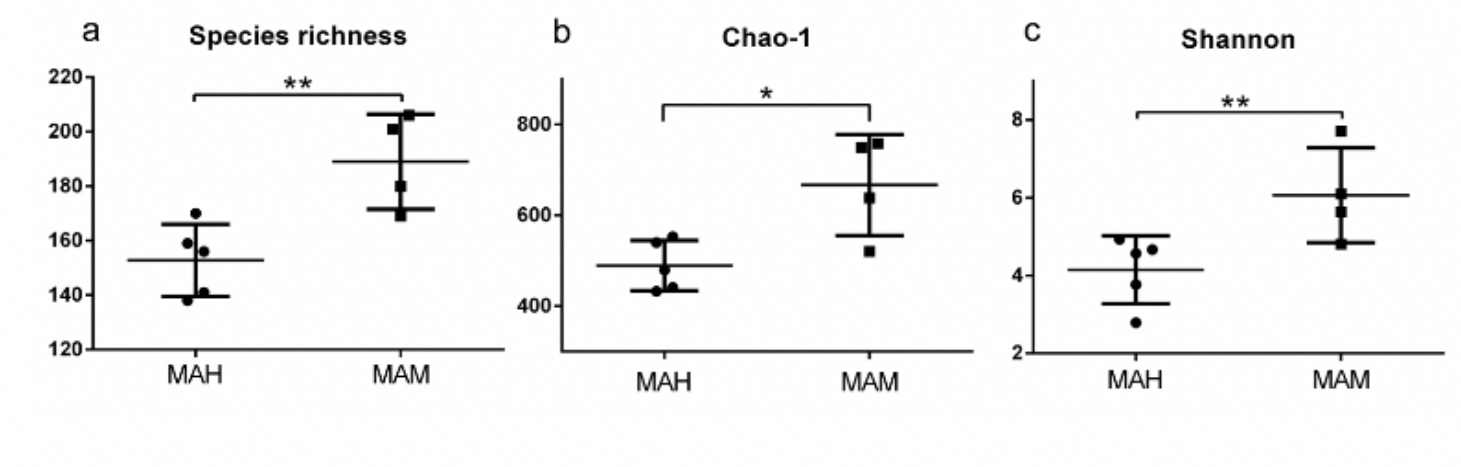

Figure 5

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. (* $p < 0.05$, ** $p < 0.01$)

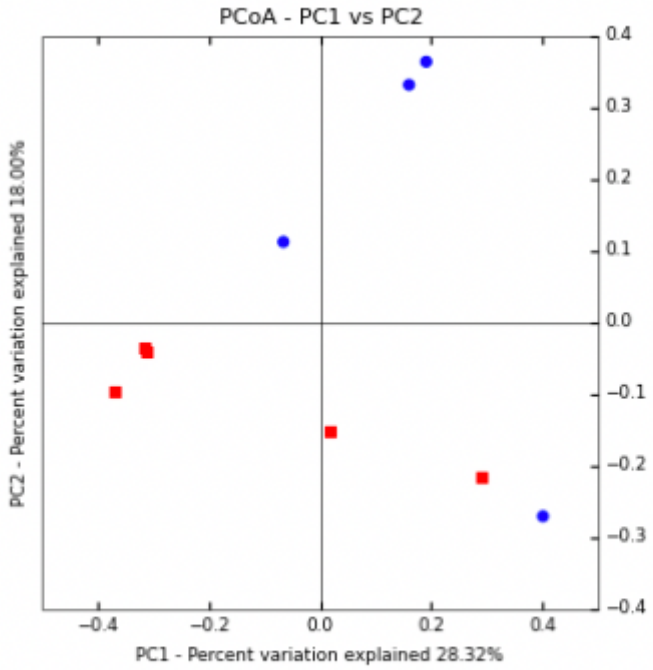

Figure 6

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.)