Subgingival Microbiome of Gingivitis in Chinese Undergraduates

Ke DENG[1], Xiang Ying OUYANG[1], Yi CHU[2], Qian ZHANG[3]

Objective: *To analyse the microbiome composition of health and gingivitis in Chinese undergraduates with high-throughput sequencing.*
Methods: *Sequencing of 16S rRNA gene amplicons was performed with the MiSeq system to compare subgingival bacterial communities from 54 subjects with gingivitis and 12 periodontally healthy controls.*
Results: *A total of 1,967,372 sequences representing 14 phyla, 104 genera, and 96 species were detected. Analysis of similarities (Anosim) test and Principal Component Analysis (PCA) showed significantly different community profiles between the health control and the subjects with gingivitis. Alpha-diversity metrics were significantly higher in the subgingival plaque of the subjects with gingivitis compared with that of the healthy control. Overall, the relative abundance of 35 genera and 46 species were significantly different between the two groups, among them 28 genera and 45 species showed higher relative abundance in the subjects with gingivitis, whereas seven genera and one species showed a higher relative abundance in the healthy control. The genera Porphyromonas, Treponema, and Tannerella showed higher relative abundance in the subjects with gingivitis, while the genera Capnocytophaga showed higher proportions in health controls. Porphyromonas gingivalis, Prevotella intermedia and Porphyromonas endodontalis had higher relative abundance in gingivitis. Among them, Porphyromonas gingivalis was most abundant.*
Conclusion: *Our results revealed significantly different microbial community composition and structures of subgingival plaque between subjects with gingivitis and healthy controls. Subjects with gingivitis showed greater taxonomic diversity compared with periodontally healthy subjects. The proportion of Porphyromonas, especially Porphyromonas gingivalis, may be associated with gingivitis subjects aged between 18 and 21 years old in China. Adults with gingivitis in this age group may have a higher risk of developing periodontitis.*
Key words: *gingivitis, high-throughput sequencing, microbiome, undergraduates*
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1 Department of Periodontology, Peking University School and Hospital of Stomatology, National Engineering Laboratory for Digital and Material Technology of Stomatology, Beijing Key Laboratory of Digital Stomatology, Beijing, P.R. China.
2 First Clinical Division, Peking University, School and Hospital of Stomatology, Beijing, P.R. China.
3 Central Laboratory, Peking University School and Hospital of Stomatology, National Engineering Laboratory for Digital and Material Technology of Stomatology, Beijing Key Laboratory of Digital Stomatology, Beijing, P.R. China.

Corresponding author: Dr Xiang Ying OUYANG, Department of Periodontology, Peking University School and Hospital of Stomatology, #22 Zhongguancun South Avenue, Haidian District, Beijing 100081, P.R. China. Tel: 86-10-82195495; Fax: 86-10-62173402. Email: kqouyangxiangy@126.com

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Gingivitis is a reversible, inflammatory disease affecting the soft tissues of the gum surrounding the teeth. Epidemiological studies have revealed that gingivitis is highly prevalent. Bleeding on probing was found in 55.7% of 12-year-olds and in 77.3% of 35 to 44 year olds in China[1]. More than 90% of US adults also suffer from gingival bleeding[2]. Longitudinal cohort study suggests that persistent gingivitis represents a risk factor for periodontal attachment loss and for tooth loss[3]. Bacteria were demonstrated as key etiologic factors in gingivitis by experimental gingivitis studies in humans[4,5]. Therefore, more attention should be paid to the bacterial profiles in gingivitis.

Previous studies of subgingival microflora of adults gingivitis showed that as gingivitis develops, the sub-

gingival microbial composition shifts from a population dominated by Gram-positive microorganisms to one with increased total number of Gram-negative anaerobes, such as Fusobacterium nucleatum and Bacteroides species[4,6,7]. For studies of oral microbiota in children and adolescents, much of the information has been obtained on the relationship of periodontopathic bacteria and gingivitis[8-12].

However, these studies have been based on microbiologic identification methods of culture, the checkerboard DNA-DNA hybridization technique, immunologically based diagnostic methods and polymerase chain reaction[4,6-12]. Those methods can be used for qualitative analysis and quantitative or half-quantitative analysis only for specific periodontopathic bacteria. However, they have limitations that do not allow microbial diversity to be fully revealed, as many low richness species cannot be detected.

High-throughput sequencing, also known as "deep sequencing of 16S rRNA gene" or "next-generation sequencing", is a landmark in the development of sequencing techniques[13,14]. The application of pyrosequencing of the 16S rRNA gene has allowed the collection of thousands of sequences per sample[15]. In the field of periodontal disease, the high-throughput sequencing method has been used to analyse oral microbiome of patients with chronic and/or aggressive periodontitis[16-19]. However, little information is available for microbial profiles of gingivitis by utilising high-throughput sequencing technique. A study of Chinese gingivitis was performed several years ago to detect oral microbiome of three gingivitis subjects via high-throughput sequencing[20]. The study of experimental gingivitis characterised the composition of plaque during the transition from periodontal health to gingivitis by pyrosequencing analysis[21]. A recent pyrosequencing analysis compared the subgingival microbiota of three distinct periodontal conditions, including health, gingivitis and periodontitis[22]. However, the average age of subjects with gingivitis in the three studies was over 21 years old[20-22]. It was believed that there was a significant effect of age on the proportions of the Red complex species, especially for Porphyromonas gingivalis, suggesting a gradual maturation of the oral microbiota[23-25]. The prevalence of Prevotella nigrescens also increased with age[26]. The period from 18 to 21 years marks the early stage of adulthood. To date, studies of oral bacteria of gingivitis in adults aged 18 between 21 years old are rare. Therefore, the aim of this study was to investigate the subgingival microbiome composition in Chinese gingivitis undergraduates aged 18 to 21 years old by utilising high–throughput sequencing technique, and to compare the bacterial profiles between these undergraduates and periodontally healthy controls.

Materials and methods

Study population

Sixty-six undergraduates aged between 18 and 21 years old were recruited from Peking University Health Science Center and enrolled on the present study. The study was approved by the Ethics Committee of the Peking University School and Hospital of Stomatology and written informed consent was obtained from each subject.

The inclusion criteria for gingivitis participants was:

- Percentage of full-mouth bleeding on probing > 20%;
- Bleeding on probing or gingival alteration, such as redness or swelling in the sampled sites;
- No attachment loss;

The inclusion criteria for periodontally healthy participants was:

- Percentages of full-mouth bleeding on probing < 10%;
- Probing depth ≤ 3 mm;
- Normal appearing, healthy gingiva in the sampled sites;
- No attachment loss.
- The exclusion criteria for all participants was:
- Wearing an orthodontic appliance;
- Having decay or restorations, such as dental prostheses in the sampled sites;
- Had a chronic medical disease or condition such as diabetes, cardiovascular disease, chronic kidney disease, hereditary disease, etc;
- Had received professional dental cleaning or antibiotic medication within the previous 3 months;
- Current smoker.

Clinical examination

Clinical parameters included: full-mouth bleeding on probing (percentage of bleeding sites was calculated), probing depth, bleeding index[27], plaque index[28], attachment level. Full-mouth bleeding on probing assessment was done as a primary screening. Two weeks later, the subjects were recalled. Firstly, supragingival plaque was removed and plaque index was recorded. Secondly, subgingival plaque samples were obtained. Finally, the remaining clinical examinations were finished. Probing depth, bleeding index and plaque index were only taken from six representative teeth: #16, #11, #26, #36, #31,

#46. Probing depth was measured at six sites per tooth: the mesiobuccal, buccal, distobuccal, distolingual, lingual, and mesiolingual positions. Bleeding index was recorded at sampled sites. Plaque index was recorded at four sites – the mesiobuccal, distobuccal, distolingual, and mesiolingual positions. The same examiner performed all clinical recordings.

Sample collection

Subgingival plaque samples were obtained from the mesiobuccal and mesiolingual sites of six representative teeth per participant, as mentioned above. After the removal of supragingival plaque and the target sites being air dried, subgingival plaque samples were obtained by inserting a sterile curette to the bottom of the gingival sulcus. In each subject, samples from 12 sites were pooled into one sterilized Eppendorf tube. The samples were stored at -80°C until used.

DNA extraction

Genomic DNA was extracted using TIANamp Bacteria DNA Kit (Tiangen Biotech, Beijing, China) from the subgingival plaque samples following the manual. The final quantity and quality of the DNA were evaluated on 1% agarose gels and a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

PCR amplification

The V3-4 hypervariable region of bacterial 16S rRNA gene were amplified with the primers 336F (5'-GTACTCCTACGGGAGGCAGCA-3') and 806R (5'- GTGGACTACHVGGGTWTCTAAT-3'). For each sample, a 10-digit barcode sequence was added to the 5'end of the forward and reverse primers (provided by Allwegene Company, Beijing, China). The PCR was carried out on a GeneAmp PCR System 9700 (Applied Biosystem, Waltham, MA, USA) using 50 μl reaction volumes, containing 5 μl 10×Ex Taq Buffer (Mg^{2+} plus) 4 μl 12.5 mM dNTP Mix (each), 1.25 U Ex Taq DNA polymerase, 2 μl template DNA, 200 nM bar-coded primers 336F and 806R each, and 36.75 μl ddH2O. Cycling parameters were 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 57°C for 30 s and 72°C for 30 s with a final extension at 72°C for 10 min. Three PCR products per sample were pooled to mitigate reaction-level PCR biases. The PCR products were purified using a QIAquick Gel Extraction Kit (QIAGEN GmbH, Hilden, Germany), quantified using Real Time PCR, and sequenced at Allwegene Company. All the DNA samples satisfied the quality and quantity standards of Allwegene Company.

Sequence processing and bioinformatic analysis

Deep sequencing was performed on the Miseq platform at Auwigene Company. After the run, image analysis, base calling and error estimation were performed using Illumina Analysis Pipeline Version 2.6. The raw data were first screened and low-quality sequences were removed. The barcodes and primers were then trimmed off. Qualified reads were analysed using QIIME (Quantitative Insights Into Microbial Ecology), version 1.8.0. The sequences were grouped into operational taxonomic units (OTUs) at a similarity level of 97% using UPARSE. The differences in richness and diversity of the species were evaluated with alpha diversity (a-diversity) indices, including Chao[1], Observed species, PD whole tree and Shannon indices. The richness of the total amount of bacteria was calculated by Chao[1] and the Observed species of OTUs. The diversity of the plaque was estimated by PD whole tree and Shannon indices. The RDP classifier 2.2 was used to assign sequences to different taxonomic groups based on the SILVA ribosomal RNA gene database[29].

Statistical analysis

Clinical and demographic data were compared via independent *t*-test, Mann-Whitney test and Chi-square test depending on the types of the data. Differences in Alpha-diversity were evaluated using independent *t*-test for healthy and gingivitis groups. To examine the differences between gingivitis and healthy group, PCA was used based on the OTU information from each sample using R version 3.31. Alpha-diversity and differences in relative abundance of individual phyla, genera and species were calculated by Mothur v.1.34.4. Analysis of similarities (ANOSIM) test on the UniFrac distances was also used to examine the community difference between healthy and gingivitis group.

Results

General information and clinical parameters

Subgingival bacterial plaque samples from 12 periodontally healthy controls and 54 subjects with gingivitis were sequenced with Illumina MiSeq technology. The demographic and clinical characteristics of subjects are depicted in Table 1. No significant differences were observed between the healthy and gingivitis groups for

Table 1 Demographic and clinical data of the study population.

	Gingivitis (n = 54)	Health (n = 12)	*P*-value
Age	19.77 ± 1.01	19.58 ± 1.16	NS[a]
Gender	22 male, 32 female,	6 male, 6 female	NS[b]
Full-mouth examination			
BOP%	32.44 (25.45; 43.60)	4.17(0.30; 6.55)	$P < 0.001$[c]
Representative teeth			
PLI	1.0(1.0;2.0)	0.5(0.0; 1.0)	$P < 0.001$[c]
PD (mm)	2.57 ± 0.88	1.98 ± 0.76	$P < 0.001$[a]
Sampled sites			
BI	2.26 ± 0.86	0	$P < 0.001$[c]

[a]Independent *t*-test; [b]Chi-square test; [c]Mann-Whitney test; Values are means ± standard deviations, median (Q25; Q75) and numbers of participants (percentage); BOP, bleeding on probing; PLI, plaque index; PD, probing depth; BI, bleeding index; NS, not significant.

age or gender. As expected, bleeding on probing, bleeding index, probing depths and plaque index were greater in the group with gingivitis than that in periodontally healthy controls.

Microbiome diversity and structure analysis

Sequencing produced 2,556,136 raw reads. 1,967,372 sequences with an average of 29,808 ± 7,612 (range 12,524-52,238) sequences per sample were obtained after initial quality filtering. In total, 286 OTUs, 14 phyla, 104 genera and 96 species-level taxa were detected across 66 samples. The mean number of OTUs for each sample was 185. First of all, we explored whether periodontal health status or gingival inflammation were related to diversity metrics. Significant differences were found in richness and diversity of subgingival plaque from gingivitis group comparing of healthy group. Chao[1], Observed species, PD whole tree and Shannon indices, known as alpha-diversity metrics were significantly higher in subgingival plaque of the gingivitis subjects compared with that of the periodontally healthy controls ($P < 0.05$) (Fig 1) Principal Component Analysis (PCA) based on OTU abundance demonstrated that gingivitis and healthy subjects were well separated, indicating distinct differences in microbiota composition between these two groups. (Fig 2). The ANOSIM test showed significant differences between subjects with gingivitis and healthy controls ($P < 0.05$).

Taxonomic analysis

Relative abundances from different taxonomic levels between subjects with gingivitis and healthy individuals were analysed, to investigate which taxa were responsible for overall community differences between health and disease. Analysis at the phylum level showed Fusobacteria, Bacteroidetes, Firmicutes, Proteobacteria, Actinobacteria, Saccharibacteria and Spirochaetae were seven of most abundant bacterial taxa. Bacteroidetes and Spirochaetae were found at higher levels in gingivitis communities, whereas other species showed no significant differences between two groups (Fig 3a). Prevalence analysis at the phylum level showed the detection rate of seven phyla was 100% both in health and gingivitis group.

At the genus level, 35 genera distributed differently between healthy and gingivitis groups, 34 genera of which had higher relative abundance in gingivitis and one genera in health. Only the genera with average relative abundance > 1% in gingivitis group or the genera with average relative abundance > 1% in healthy group were showed in Fig 3b. Prevalence analysis at the genus level showed the detection rate of these genera was 100%. Porphyromonas (mean 8.31%), Treponema 2 (mean 3.18%), Tannerella (mean 1.13%) and Bacteroidales genomosp (mean 1.03%) showed higher abundances in the gingivitis, whereas the genera Capnocytophaga had higher abundances in health.

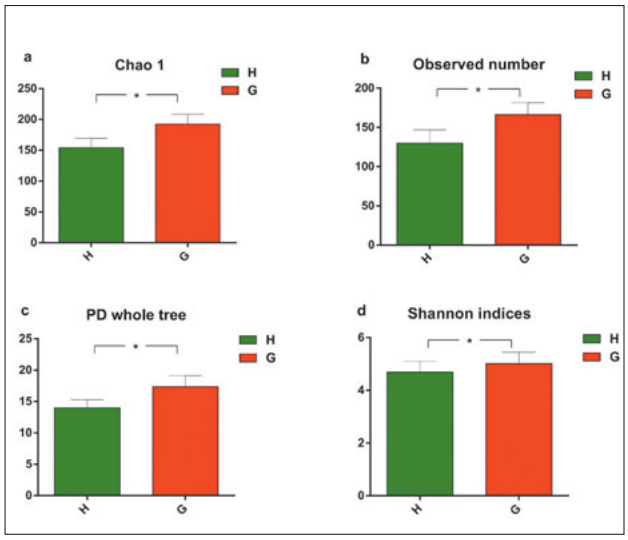

Fig 1 α-diversity of plaque samples in gingivitis group and periodontally healthy group. Chao[1] a) Observed species; b) PD whole three; c) and Shannon indices; d) in subgingival plaque of the subjects with gingivitis and periodontally healthy controls. H, healthy group; G, gingivitis group.

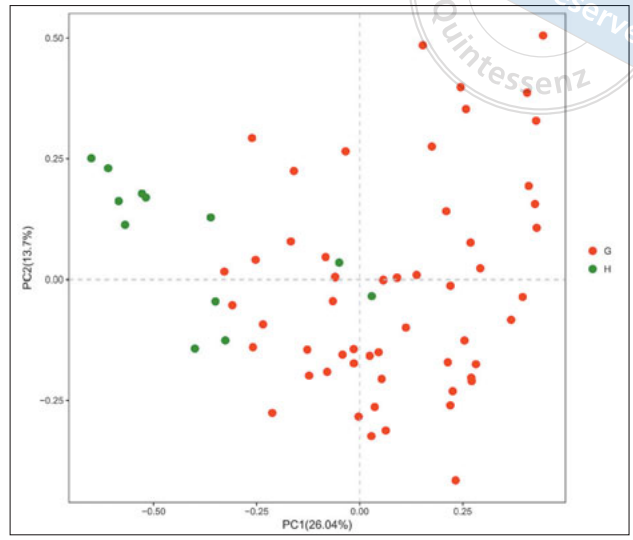

Fig 2 PCA plot based on OTU abundance of subgingival microbiota in health and gingivitis. Communities from gingivitis cluster apart from those in health. Communities from healthy subjects were coloured green, whilst communities from subjects with gingivitis were coloured red.

At the species level, a total number of 46 species had different distribution in subgingival plaque between healthy and gingivitis groups, 45 species of which had higher relative abundance in gingivitis and one species in health. Only the species with average relative abundance > 0.5% in gingivitis group was showed in Fig 3c. No difference in prevalence of these species between health and gingivitis was detected. The relative abundance of Fusobacterium nucleatum was greatest both in gingivitis and healthy group. The abundance of Porphyromonas gingivalis W83 (mean 5.90%), Prevotella intermedia 17 (mean 2.16%) and Porphyromonas endodontalis (mean 1.88%) were significantly higher than those in healthy controls. Among them, Porphyromonas gingivalis (mean 5.90%) was the most abundant specie in the subgingival plaque samples from gingivitis subjects and Porphyromonas gingivalis was detected in all subjects. The detection rate of Prevotella intermedia 17 and Porphyromonas endodontalis has no significant difference. No difference in relative abundance of Capnocytophaga spp. between the two groups was detected. The relative abundance of Aggregatibacter actinomycetemcomitans was relatively low both in gingivitis (mean 0.16%) and healthy groups (mean 0.007%). Aggregatibacter actinomycetemcomitans was detected in 87% of the gingivitis subjects and 3.7% of the healthy controls.

Most bacterial taxa were detected both in health and gingivitis. However, 1 phylum and 13 genera, which

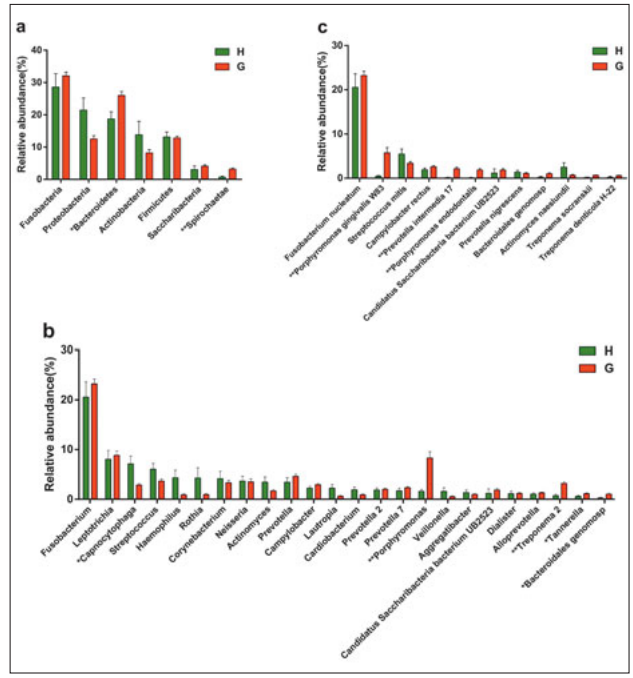

Fig 3 Differences between healthy (H) and gingivitis (G) subjects at the phylum, genus and species levels. a) Phylum level. The phyla with average relative abundance > 0.4% are present; b) Genus level. The genera with average relative abundance > 1% in the gingivitis group or the genera with average relative abundance > 1% in healthy group are present; c) Species level. The species with average relative abundance > 0.5% in the gingivitis group is present. Bars represent the mean (± SD) relative abundance of phyla, genera and species. * Indicates a $P < 0.05$ and ** indicates a $P < 0.01$.

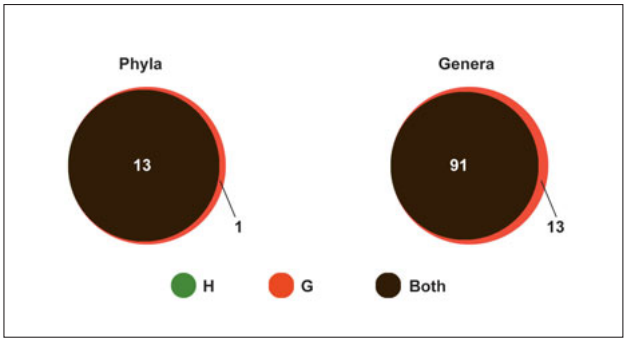

Fig 4 Venn diagram of healthy and gingivitis communities. The figure shows the intersection for two communities at the level of phylum and genus. H, healthy; G, gingivitis.

accounted for quite low relative abundance, were only found in gingivitis (Fig 4). Among them, we found one phylum and six genera with significantly different relative abundance. Those genera included: Wolinella (25/54, detection rate of 46.3%, $P < 0.05$), Bacteroides (14/54, detection rate of 25.9%, $P < 0.05$), Bulleidia 22/54, detection rate of 40.7%, $P < 0.05$, Prevotellaceae UCG 00416/54, detection rate of 29.6%, $P < 0.05$, Eggerthia (13/54, detection rate of 24.1%, $P > 0.05$) and Proteiniphilum2/54, detection rate of 3.7%, $P > 0.05$.

Discussion

Recent advances in DNA sequencing and bioinformatics technologies have provided more information for the analysis of the oral bacterial microbiome in periodontal disease[16,18,19,30]. However, studies of subgingival bacteria in gingivitis by utilising high-throughput sequencing technique are rare. In this study, we revealed a holistic view of subgingival microbial communities in subjects with health and gingivitis aged 18 to 21 years old by Illumina MiSeq sequencing technologies, which showed distinct differences in community composition between the two groups. Besides confirming previous findings that certain species are more prevalent in disease, this work provides a broader picture of overall community differences.

This study revealed two groups of microorganisms distributed differently between the healthy and gingivitis groups. The first group was the common species shared by health- and gingivitis-associated communities, but had different relative abundance between two communities. For example, some disease-associated taxa were also present in health, but accounted for a smaller fraction of the total community. Among these common species shared by the health and gingivitis communities, a total of 28 genera had higher relative abundance in gingivitis. The second group was the species, which were only detected in the gingivitis group. There were only six genera detected in gingivitis, but these microorganisms accounted for a fairly low proportion. Among them, the genera Wolinella, Bacteroides, Bulleidia and Prevotellaceae UCG 004 were more frequently detected in gingivitis, while Eggerthia and Proteiniphilum had fairly low frequency detection. Perhaps these little-studied species seemed more likely to play a role in the pathogenesis of gingivitis. Further research should pay more attention to these species.

This study also provided an overall view of subgingival microbial communities in health and gingivitis from different taxonomic levels. Our study demonstrated that the genus Porphyromonas (mean 8.31%) significantly increased in the gingivitis group, showing an apparent association with disease. Similarly, the genus Porphyromonas (mean 37.75%) was found to be the most abundant genera in the subgingival plaque samples from aggressive periodontitis patients in China[19]. Interestingly in our study, the genus Porphyromonas (mean 1.61%) was also detected in healthy subjects in our study. We speculate that the proportion of the genus Porphyromonas may play a role in disease progression, such as gingivitis and periodontitis. Moreover, we observed a remarkably higher level of Porphyromonas gingivalis (mean 5.90%), Prevotella intermedia (mean 2.16%) and Porphyromonas endodontalis (mean 1.88%) in the gingivitis group, indicating that these microorganisms, especially for Porphyromonas gingivalis are associated with gingivitis. Consistent with these results, previous findings also demonstrated that the detection of Porphyromonas gingivalis and Prevotella intermedia was significantly higher in the gingivitis group[31].

Chen et al considered that Porphyromonas gingivalis was one of the most important periodontopathic bacteria in gingivitis during puberty[32]. Indeed, Porphyromonas gingivalis is well known as gram-negative, anaerobic bacterium involved in the pathogenesis of periodontitis, which are often found in deep periodontal pockets[33,34]. Prevotella intermedia are a gram-negative, obligate anaerobic pathogenic bacterium involved in periodontal infections, including gingivitis and periodontitis, and often found in acute necrotizing ulcerative gingivitis[35]. Lourenço et al also found that detection of Porphyromonas endodontalis was a risk indicator of periodontal disease[36]. Therefore, it is likely that the gingivitis subjects aged between 18 and 21 in this study have a significantly higher risk of developing periodontitis. In addition, our study suggested that the Bacteroidetes was the

dominant phyla and had a remarkably higher level in gingivitis subjects. As with our observations, previous studies suggest that the relative abundance of the phyla Bacteroidetes was significantly higher in the gingivitis group compared with healthy controls[21,22]. However, we also found that Spirochaetae showed significantly higher relative abundance in gingivitis, while Huang et al found no significant difference between health and gingivitis[20]. Moreover, our gingivitis communities showed higher abundance of Spirochaetae than those studied by Huang et al[20]. This discrepancy could be explained by different inflammatory conditions of subjects. The average percentage of full-mouth bleeding on probing of subjects with gingivitis in the study of Huang et al. was 29%[20]. Indeed, more than 63% of subjects had higher percentages of full-mouth bleeding on probing over 29% in our study, indicating more severe inflammatory state of our subjects with gingivitis. The result was supported by the previous study. It was reported that Spirochaetae could overgrow in the gingivitis plaque and was associated with a severe inflammatory response[37].

An interesting discovery in this study is a remarkably higher relative abundance of the genus Capnocytophaga in health, while previous results revealed significant higher level of Capnocytophaga in gingivitis community[22]. This discrepancy may be explained by differences in age, ethnicity and geographic region. To date, only six members of this genus (Capnocytophaga gingivalis, Capnocytophaga ochracea, Capnocytophaga sputigena, Capnocytophaga granulosa and Capnocytophaga haemolytica, Capnocytophaga leadbetteri) have been isolated from subgingival plaque[38,39]. It was reported that Capnocytophaga gingivalis was associated with gingivitis and Capnocytophaga ochracea was found in higher proportion in the subgingival plaque of subjects with periodontitis[40]. The clinical significance of Capnocytophaga granulosa and Capnocytophaga haemolytica in subgingival plaque has not as yet been ascertained[39]. In fact, the relative abundance of Capnocytophaga species known to us found no significant difference between the health and gingivitis groups. It hints that maybe some unknown Capnocytophaga *spp.* is associated with health.

In conclusion, this study, using high-throughput sequencing techniques, revealed significantly different microbial community composition and structures of subgingival plaque between gingivitis and healthy subjects. Subjects with gingivitis showed greater taxonomic diversity, compared with periodontally healthy subjects. The proportion of Porphyromonas, especially Porphyromonas gingivalis, may be associated with gingivitis subjects aged between 18 and 21 years old in China. Adults with gingivitis in this age group may have a higher risk of developing periodontitis. Therefore, more attention should be paid to the prevention and treatment of gingivitis.

Conflicts of interest

The authors reported no conflicts of interest related to this study.

Author contribution

Dr Ke DENG designed and performed the experiments and prepared the manuscripts; Dr Xiang Ying OUYANG designed the study and finally approved the manuscript; Dr Yi CHU designed and performed the study; Dr Qian ZHANG provided technique instruction.

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