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Supragingival microbiome variations and the influence of *Candida albicans* in adolescent orthodontic patients with gingivitis

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ABSTRACT

Introduction: Gingivitis is a prevalent complication in adolescents undergoing fixed orthodontic treatments. However, changes in the supragingival microbiome associated with gingivitis and the impact of *Candida albicans* remain elusive. Therefore, we investigated supragingival microbiome discrepancy and *C. albicans* colonization in adolescent orthodontic patients with gingivitis.

Methods: Dental plaques were collected from 30 gingivitis patients and 24 healthy adolescents, all undergoing fixed orthodontic treatment. The supragingival microbiome composition was analyzed using 16S rRNA sequencing. *C. albicans* colonization was determined using fungal culture and real-time quantitative polymerase chain reaction.

Results: Our analysis revealed significantly heightened microbial diversity in the Gingivitis group. Notably, patients with gingivitis exhibited an enrichment of periodontal pathogens, such as *Saccharibacteria (TM7) [G-1]*, *Selenomonas*, *Actinomyces dentalis*, and *Selenomonas sputigena*. Additionally, 33% of the gingivitis patients tested positive for *C. albicans*, exhibiting significantly elevated levels of absolute abundance, while all healthy patients tested negative. Significant differences in microbial composition were also noted between *C. albicans*-positive and -negative samples in the Gingivitis group.

Conclusion: Significant disparities were observed in the supragingival microbiome of adolescent orthodontic patients with and without gingivitis. The presence of *C. albicans* in the supragingival plaque may alter the microbiome composition and potentially contribute to gingivitis pathogenesis.

KEY MESSAGES

- Adolescent patients undergoing fixed orthodontic treatment, with and without gingivitis, show significant differences in their marginal supragingival plaque microbiomes.
- Adolescent patients with gingivitis exhibit a significantly higher rate of *Candida albicans* colonization than healthy individuals.
- The colonization of *C. albicans* alters the composition of the marginal supragingival plaque microbiome in patients with gingivitis.

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Fixed orthodontic treatment; gingivitis; supragingival plaque; microbiome diversity; *candida albicans*

Introduction

Marginal gingivitis is a common side effect of orthodontic treatment and a major biofilm-related disease that affects treatment effectiveness [1]. Compared to adult patients, adolescents are less diligent in maintaining oral hygiene, leading to a higher incidence of marginal gingivitis [2]. Orthodontics-related gingivitis affects up to 56.8% of adolescents [3]. When the periodontal tissue is in an inflammatory state, the formation and activity of osteoclasts increase during tooth movement, which can worsen alveolar bone loss, posing risks to periodontal tissue and dental health [4].

Dental plaque and its byproducts that accumulate at the gingival margin are key factors in developing marginal gingivitis. Consequently, there is a growing focus on exploring changes in dental plaque associated with marginal gingivitis during fixed orthodontic treatment. Previous studies using bacterial culture techniques have shown a substantial increase in Gram-negative bacteria in the dental plaque of patients with orthodontic gingivitis [5]. Recent advancements in high-throughput techniques have enabled the exploration of changes in the gingival microbiome during orthodontic treatment [6–8]. Previous research on gingival inflammation during orthodontic treatment primarily examined subgingival plaque or saliva. Studies have shown that orthodontic treatment may increase plaque adhesion and alter the subgingival microbiome [9], leading to mild gingival inflammation [10]. It has been confirmed that microbial diversity in subgingival plaque typically increases in patients with fixed orthodontic treatment [11]. Specifically, *Tannerella forsythia* and *Prevotella intermedia* have been found to increase significantly in subgingival plaque, indicating an increased risk of

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periodontal infection during orthodontic treatment [12]. In saliva samples, the microbial compositions of orthodontic patients differed considerably from those of healthy individuals, with orthodontic patients exhibiting higher microbial diversity [7]. While most studies have focused on subgingival plaque or saliva, a few have examined changes in supragingival marginal plaque during orthodontic treatment [13]. These studies found an increase in anaerobic bacteria; however, participants did not exhibit typical symptoms of gingivitis [13]. Research on microbiome changes in orthodontic patients with typical gingivitis, particularly among adolescents, who represent a significant portion of orthodontic patients, remains limited.

In addition to bacteria, fungi are important constituents of the oral microbiome and can contribute to the pathogenesis of various diseases [14–16]. In particular, *Candida* is closely linked to oral diseases. There are over 150 *Candida* species, with approximately 20 identified as human pathogens that can interact with bacteria to cause disease [17]. In the context of periodontal disease, research on *Candida albicans* has mainly focused on patients with periodontitis, and a notable positive correlation between *C. albicans* and the development of periodontal disease has been reported [18]. *C. albicans* has been found in the subgingival plaques of approximately 29.8% of individuals with chronic periodontitis [19]. Furthermore, observations have indicated that *C. albicans* hyphae can infiltrate the periodontal connective tissue [20], interact with subgingival bacterial pathogens, or induce proinflammatory cytokine production, thereby leading to the loss of periodontal attachment and aggravating periodontal disease [18]. *C. albicans* can also create an anoxic microenvironment in biofilms, supporting the growth of anaerobic bacteria [21]. For example, *C. albicans* biofilm can protect the anaerobic bacterium *Porphyromonas gingivalis* from the aerobic environment [21]. However, most studies have primarily investigated the presence of *C. albicans* in subgingival plaques of individuals diagnosed with chronic periodontitis; however, only a few studies have detected *C. albicans* in supragingival plaques of adult patients with gingivitis [22]. Moreover, the enrichment of *C. albicans* in adolescent patients with marginal gingivitis during orthodontic treatment and its potential relationship with bacterial abundance require further exploration.

Therefore, we investigated whether the composition of the supragingival microbiome of healthy adolescent patients undergoing orthodontic treatment differs from that of patients with gingivitis through 16S rRNA sequencing. Additionally, we utilized fungal culture, rDNA internal transcribed spacer identification (ITS) and real-time quantitative polymerase chain reaction (qPCR) to investigate whether *C. albicans* enrichment occurs in gingivitis and to explore the impact of *C. albicans* aggregation on bacterial composition, thereby providing further evidence concerning the physiological and ecological significance of the supragingival plaque microbiome in the development of orthodontic gingivitis. The null hypotheses for this research were as follows: (1) There is no statistically significant difference observed in the supragingival microbial composition between orthodontic patients with gingivitis and healthy individuals; (2) *C. albicans* is not enriched in adolescents with gingivitis; and (3) *C. albicans* enrichment does not affect the supragingival plaque microbiome composition.

Materials and methods

Recruitment of patients

This study obtained ethical approval from the Ethics Committee of the Beijing Stomatological Hospital. All participants included in this study obtained the consent of the patients and their parents and signed informed consent for the study. Adolescents aged 11–18 years undergoing fixed orthodontic treatment were enrolled from Beijing Stomatological Hospital, involving 30 patients with gingivitis (the Gingivitis group) and 24 patients without gingivitis as the control group (the Periodontal healthy group). In the Gingivitis group, participants had gingivitis in both anterior teeth and premolars with an average gingival index (GI) $\geq$ 1, attachment loss $\leq$1 mm, and DMFS index < 10 [23,24]. The inclusion criteria for the Periodontal healthy group were periodontal health, GI < 0.5, PD $\leq$3 mm, DMFS index < 10, and no periodontal attachment loss.

Dental plaque collection

Participants were instructed to abstain from oral hygiene activities for at least 12 h before sample collection and to avoid eating or drinking for 2 h prior to sampling. Using a periodontal curette, supragingival plaque along the gingival margin of the anterior teeth and premolars was collected.

The plaque samples were then placed into 1 mL of sterilized TE buffer and transported to the laboratory on ice. The collected plaque was divided into two parts. One aliquot underwent bacterial 16S rRNA sequencing analysis and quantification of *C. albicans*, while the other aliquot was preserved in 1× TE buffer (containing 20% glycerol) for subsequent *Candida* culture identification and ITS sequencing analysis. All samples were stored at −80°C for future use.

Genomic DNA extraction, illumina MiSeq sequencing, and processing

Genomic DNA was extracted from 54 samples using the FastDNA Spin Kit (MP Biomedicals, USA)

following the manufacturer's instructions. The quality and concentration of DNA samples were assessed using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). The V1-V3 region of 16S rRNA fragments was amplified with primers 27F and 533 R, which were 5' -AGAGTTTGAT CCTGGCTCAG-3' and 5'- TTACCGCGGCTGCT GGCAC-3' respectively. The PCR protocol comprised an initial denaturation at 95°C for 3 min, followed by 27 cycles of denaturation at 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s, and a final extension step at 72°C for 10 min. The amplicons were paired-end sequenced using the Illumina MiSeq platform with PE300. The raw sequencing reads were deposited in the NCBI Sequence Read Archive database under the accession number SRP483771.

After demultiplexing, we used FLASH (v1.2.7) to merge the obtained sequences [25] and Fastp (v0.19.6) for quality filtering [26]. The parameters were set as follows: (1) Trim low-quality bases from the end of reads with a minimum quality threshold of 20; use sliding window trimming with a 50 bp window; discard reads under 50 bp in length or containing N bases. (2) Pairwise reads with overlapping PE reads were merged into a sequence, and the minimum overlap length was 10 bp. (3) The maximum mismatch ratio in the overlap region of the splicing sequence was 0.2. (4) Samples were identified by barcode and primer sequences at both ends, allowing zero mismatches for barcodes and up to two for primers. Using the DADA2 [27] plug-in within the Qiime2 pipeline, sequences were denoised under the following conditions [28]: discard reads with a length less than or equal to zero; remove sequences with a total abundance below ten across all samples or less than two in any single sample. The MaxEE was set at two, and the truncQ at zero. Post-denoising, amplicon sequence variants were identified and assigned taxonomic classifications using the Qiime2 naïve Bayes consensus taxonomic classifier and the Human Oral Microbiome Database (HOMD) (v15.2) [29].

Culture, identification, and quantification of C. albicans

Dental plaque samples and *C. albicans* (SC5314) used to establish standard curves were inoculated onto CHROMagar selective culture medium (Becton Dickinson & Co., USA) and incubated at 37°C for 72 h [30]. *C. albicans*-positive colonies, which appear as green colonies on CHROMagar *Candida*-selective medium [31], were then collected using a sterile inoculation ring and transferred to 2 mL sterile centrifuge tubes for subsequent DNA extraction and analysis of the rDNA ITS sequences [31].

The total genomic DNA was extracted using Epicenter MasterPure DNA extraction kits (Lucigen Corporation, USA), following the manufacturer's instructions. The total volume of the PCR amplification mixture was 50 μL, including enzyme-free water (17 μL), 2 × Taq PCR Mastermix (25 μL) (KT201, Tiangen Biotechnologies, China), template DNA (4 μL), and ITS primers (2 μL). The amplification of the extracted DNA was performed using primers ITS4 and ITS5, which were 5' - TCCTCCGCTTA TTGATATGC – 3' and 5' - GGAAGTAAAAGT CGTAACAAGG – 3' respectively. The sample denaturation was conducted at 95°C for 5 min, followed by 35 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 60 s, and a final extension step at 72°C for 10 min [32].

The amplified products were sequenced by the Beijing Genomics Institution to identify fungal strains. The PCR products were initially detected on a 1.0% agarose gel using 3 μL of the PCR products and subsequently purified following the standard operating procedure for magnetic bead purification. This process leverages the principle that magnetic beads can absorb or release charged substances – DNA is adsorbed in a high-salt, low-pH solution, facilitating DNA separation and purification. The purified PCR products were sequenced using an ABI 3730 sequencer (Applied Biosystems, Inc, USA). BioEdit and Cexpress software were used for sequence assembly and correction. The obtained sequences were then subjected to comparative analysis against homologous sequences available using the BLAST software in the GenBank database. Strains showing high sequence similarity were selected for further study. The raw sequencing reads have been deposited in the NCBI GenBank database under the accession numbers PP563750-PP563769.

To quantify the abundance of *C. albicans* in the samples, we performed a real-time quantitative polymerase chain reaction. We extracted and purified the total genomic DNA from the *C. albicans* standard strain and another aliquot of the supragingival plaque, as described in section 2.3. We used *C. albicans*-specific primers CALB1 (5' - TTTATCAACT TGTCACACCAGA – 3') and CALB2 (5' - ATCCCGC CTTACCACTACCG – 3') for the qPCR reactions, which were conducted on a Bio-Rad CFX Connect (BioRad, Hercules, CA, USA). The protocol included denaturation at 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. We determined the quantification of *C. albicans* in each sample by converting DNA concentrations to colony-forming units (CFU/mL) using a standard curve.

Statistical analysis

Four diversity indices – ACE, Chao1, Simpson, and Shannon – were selected to evaluate the alpha

diversity in this study. Beta diversity was analyzed through principal coordinate analysis (PCoA) using the Bray-Curtis distance matrix, and the ADONIS test was applied to assess statistical distinctions. Both alpha and beta diversity analyses were performed on the Majorbio Cloud Platform. The Wilcoxon rank-sum test was used to assess differences in taxonomic composition between groups, whereas the Kruskal-Wallis test was used to compare the absolute abundance of *C. albicans* across different groups. A two-tailed p value of < 0.05 was considered statistically significant.

Results

Significant differences in the marginal supragingival plaque microbiome between the gingivitis and periodontal healthy groups

This study compared the differences in supragingival microbiota between adolescents undergoing fixed orthodontic treatment with typical gingivitis and those with periodontally healthy status (Table 1). All patients in the Gingivitis group met the diagnostic criteria for moderate gingivitis, as indicated by GI values ranging from 1.1 to 1.9 [23]. Sequencing analysis yielded 4,335,107 raw reads from the 54 supragingival plaque samples. Subsequent quality filtration yielded 4,083,635 optimized sequences, with an average length of 482 base pairs. Denoising the optimized sequences using the DADA2 plug-in in the Qiime2 pipeline yielded 1,148,723 sequences. Finally, 12 phyla, 29 classes, 48 orders, 79 families, 144 genera, and 482 species were detected through high-throughput sequencing. The alpha diversity of the supragingival microbiome of the two groups was analyzed to evaluate differences in species richness and diversity (Figure 1a). The Shannon index was significantly higher in the Gingivitis group than in the Periodontal healthy group ($p < 0.05$), indicating a significantly higher diversity of the periodontal microbiome in the Gingivitis group. This result corresponded with the previous findings, showing that inflamed periodontal tissue is associated with increased microbial diversity and a more complex microbiome community structure [10]. The PCoA of the supragingival microbiome composition indicated that the beta diversity of the Gingivitis and Periodontal healthy groups was significantly different from each other ($p < 0.05$) (Figure 1b).

We further explored the differences in bacterial abundance in the supragingival microbiome between the two groups by identifying the core microbiota with a relative abundance of more than 1.0% at the genus level. The results showed that the core genus compositions of the two groups were similar. As shown in Figure 2a, the ten dominant genera included *Leptotrichia*, *Streptococcus*, *Saccharibacteria (TM7)*

Table 1. Mean (standard deviation) of basic information and clinical indicators.

Grouping information	Age	Course of treatment (month)	GI	DMFS
Gingivitis (n = 30)	14.17 (1.60)	9.83 (1.62)	1.15 (0.12)	2.57 (1.10)
Periodontal healthy (n = 24)	14.58 (1.52)	9.75 (1.72)	0.26 (0.11)	2.67 (1.01)

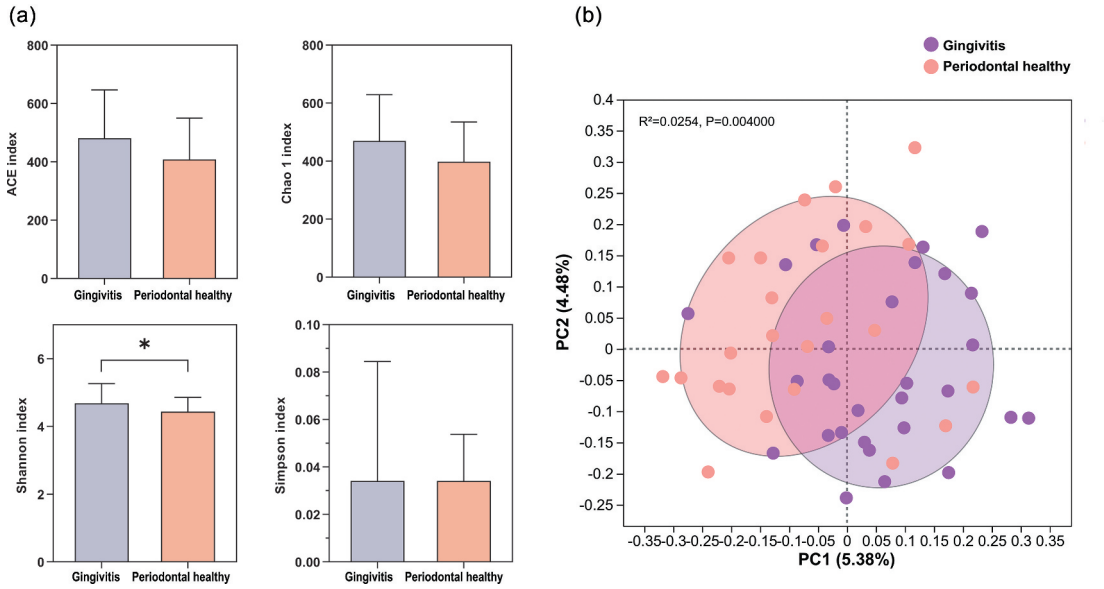

Figure 1. Comparison of alpha and beta diversities of microbial communities between the Gingivitis group and Periodontal healthy group. (a) Four indices, ACE, Chao1, Simpson, and Shannon, were selected to compare the alpha diversity (*p < 0.05). (b) Principal Coordinate Analysis (PCoA) was used to analyze beta diversity, and the ADONIS test was applied to evaluate statistical distinctions.

[G-1], *Actinomyces*, *Selenomonas*, *Capnocytophaga*, *Prevotella*, *Corynebacterium*, *Veillonella*, and *Fusobacterium*. The Gingivitis group exhibited a high relative abundance of *Saccharibacteria (TM7) [G-1]*, *Selenomonas*, and *Prevotella*; however, *Capnocytophaga*, *Neisseria*, *Lautropia*, and *Rothia* exhibited significant enrichment in the Periodontal healthy group ($p < 0.05$) (Figure 2b). Several TM7 species like *Saccharibacteria (TM7) [G-1] bacterium HMT 346* and *Saccharibacteria (TM7) [G-5] bacterium HMT 356*; bacteria of the *Streptococcus* genus, such as *Streptococcus oralis subsp._tigurinus_clade_071*, and *Streptococcus anginosus*; as well as bacteria of other genera, such as *Actinomyces dentalis* and

Figure 2. Species composition analysis between Gingivitis group and Periodontal healthy group. (a) Microbial composition at the genus level between the Gingivitis group and Periodontal healthy group. (b) The genera with significant differences between the Gingivitis group and the Periodontal healthy group (taking the top 1–15 bacteria ranged by relative abundance). (c) The species with significant differences between the Gingivitis group and the Periodontal healthy group (taking the top 1–25 bacteria ranged by relative abundance). (d) Periodontal pathogens with low relative abundance but significant differences between the Gingivitis group and Periodontal healthy group (*p < 0.05, *p < 0.01, **p < 0.001).

Selenomonas sputigena, also exhibited significant enrichment in the Gingivitis group at the species level ($p < 0.05$) (Figure 2c). Furthermore, dominant bacteria found in gingivitis patients reported by previous studies, such as *P. intermedia* and *Fusobacterium nucleatum*, and bacteria typically detected in chronic periodontitis, such as *T. forsythia* and *Treponema denticola*, were also enriched in the Gingivitis group (Figure 2d). However, *Capnocytophaga granulosa*, *Lautropia mirabilis*, *Streptococcus sanguinis*, *Actinomyces sp. HMT_169*, *Capnocytophaga sputigena*, *Cardiobacterium hominis*, *Actinomyces massiliensis*, *Neisseria elongata*, *Rothia aeria*, and *Corynebacterium durum* were significantly enriched in the Periodontal healthy group ($p < 0.05$) (Figure 2c).

The gingivitis group exhibits a high C. albicans colonization rate and load

C. albicans colonization was initially identified by inoculating the plaque sample on CHROMagar *Candida*-selective culture medium, and positive colonies were confirmed through ITS sequencing. No *C. albicans*-positive colonies emerged on CHROMagar *Candida*-selective medium inoculated with samples from the Periodontal healthy group; however, ten out of the 30 samples from the Gingivitis group yielded green colonies. ITS sequencing results confirmed that all ten *Candida*-positive samples in the Gingivitis group were *C. albicans*, indicating a colonization rate of up to 33% in the Gingivitis group (Figure 3a). The detection rate of *C. albicans* was higher in the Gingivitis group than in the Periodontal healthy group, suggesting that *C. albicans* colonization is more likely in patients with gingivitis than in healthy patients. Based on the detection rates of *C. albicans*, we classified the Gingivitis group into two subgroups: Gingivitis-*C. albicans* positive (G-CaP, $n = 10$) and Gingivitis-*C. albicans* negative (G-CaN, $n = 20$). As shown in Figure 3b, the abundance of *C. albicans* in the G-CaP group was significantly higher than that in the G-CaN group and Periodontal healthy groups ($p < 0.01$).

C. albicans influences the supragingival microbiome composition in patients with gingivitis

To understand the effect of *C. albicans* on the supragingival microbiome of gingivitis plaque, we further performed the taxonomic analysis between the G-CaP ($n = 10$) and G-CaN ($n = 20$) groups. No statistical differences were observed in alpha diversity between the two subgroups ($p > 0.05$) (Figure 4a); however, the PCoA of the supragingival microbiome composition showed that the beta diversity of the two groups was significantly different from each other ($p < 0.05$), indicating that the microbial community composition of the G-CaP group was significantly different from that of G-CaN samples (Figure 4b). Further investigation of the differences in bacterial abundance in the supragingival microbiome of the G-CaP and G-CaN groups indicated that the core genera of the two groups were similar; however, the G-CaP group exhibited a greater abundance of *Leptotrichia*, *Selenomonas*, and *Prevotella* than the G-CaN group (Figure 5a). Furthermore, the difference in taxonomic composition showed that *Campylobacter* was more abundant in the G-CaP group, whereas *Saccharibacteria (TM7) [G-5]* and *Treponema* were more abundant in the G-CaN group ($p < 0.05$) (Figure 5b).

Species-level bacteria related to periodontal diseases, such as *S. noxia*, *Campylobacter gracilis*, *Actinomyces sp._HMT_448*, *Campylobacter concisus*, *Prevotella salivae*, *Actinomyces oris*, and *Veillonella atypica*, were enriched in the G-CaP group ($p <$

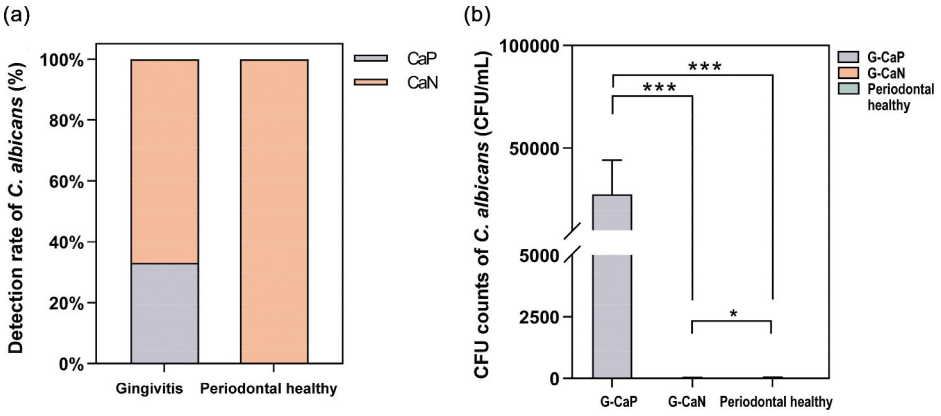

Figure 3. (a) the difference of *Candida albicans* detection rate between Gingivitis group and Periodontal healthy group. CaP: *C. albicans-positive*; CaN: *C. albicans-negative*. (b) The CFU counts of *C. albicans* in G-CaP, G-CaN, and Periodontal healthy groups (NS: no significance, *p < 0.05, *p < 0.01, **p < 0.001).

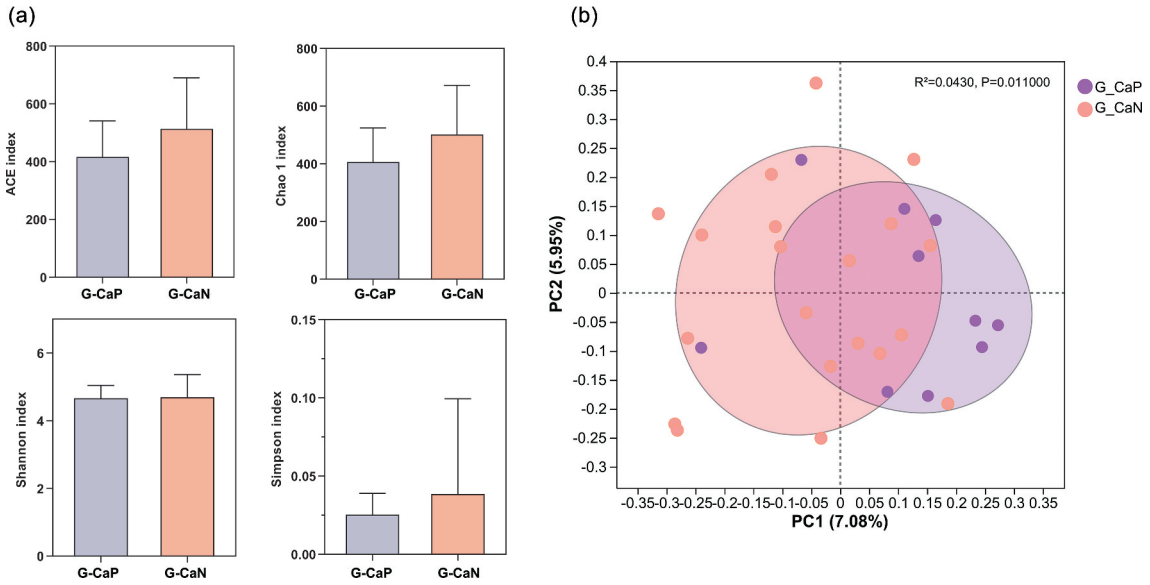

Figure 4. Comparison of alpha and beta diversities of microbial communities between the G-CaP and G-CaN group. (a) Four indices, ACE, Chao1, Simpson, and Shannon, were selected to compare the alpha diversity (*p < 0.05). (b) Principal Coordinate Analysis (PCoA) was used to analyze beta diversity, and the ADONIS test was applied to evaluate statistical distinctions.

0.05) (Figure 5c). However, the abundance of *TM7 species HMT-356*, *Gemella morbillorum*, *Leptotrichia sp._HMT_225*, and *L. mirabilis* were significantly increased in the G-CaN group ($p < 0.05$). Additionally, although their abundance was relatively low, some periodontal pathogens are more commonly detected in subgingival plaque. For example, *F. nucleatum* subsp. *nucleatum* and *Aggregatibacter actinomycetemcomitans* were only detected in the G-CaP group (Figure 5d).

Discussion

Gingivitis is a common complication of orthodontic treatment that affects the periodontal tissue. In order to clarify the role of the oral microbiome in the pathogenesis of adolescent gingivitis, we investigated the disparities in the oral microbiome between adolescent orthodontic patients with and without gingivitis, as well as the impact of *C. albicans* for the first time. Our results showed significant differences in the supragingival microbiome between adolescents with gingivitis and those with a healthy status. Furthermore, all three invalid hypotheses were rejected.

During orthodontic treatment, the accumulation of dental plaque near the gingival margin and direct stimulation by orthodontic appliances can lead to gingivitis. Dental plaque and its byproducts at the gingival margin are pivotal in developing marginal gingivitis. Fixed orthodontic appliances, such as brackets and bands, complicate oral hygiene and aggravate plaque buildup [33]. Additionally, the metal components in these appliances, such as nickel alloys, can be toxic to oral bacteria and may prompt gingival overgrowth by increasing epithelial cell proliferation due to continuous low-dose nickel release [34]. Furthermore, nickel ions exert toxic effects on oral bacteria, contrasting with other metal like iron, which are essential for the metabolism of bacteria such as *P. intermedia* [35,36]. Therefore, beyond promoting plaque accumulation, orthodontic appliances may also disturb the ecological balance of the supragingival microbiome. Furthermore, orthodontic forces can elevate matrix metalloproteinase 8 levels, potentially contributing to gingival hyperplasia in orthodontic patients [37]. Additionally, during certain movements like intrusion or tipping, plaque can migrate into subgingival areas, intensifying periodontal inflammation [12]. Therefore, given that dental plaque is the primary factor in developing marginal gingivitis, gingivitis associated with orthodontic treatment exhibits distinct characteristics compared to gingivitis without orthodontic intervention. This specificity is also confirmed by the differences in microorganisms found in orthodontic versus non-orthodontic gingivitis. Research has shown significant variations in the periodontal pathogens present in subgingival plaque between patients with fixed orthodontic appliances and those without. Notably, the presence of *T. forsythia*, *T. denticola*, and *P. nigrescens* is markedly elevated in orthodontic patients, suggesting that changes associated with fixed orthodontic appliances may influence the prevalence of these periodontal pathogens in subgingival plaques [38]. Another study revealed an 85% detection rate of *A. actinomycetemcomitans* in the subgingival plaque of adolescents undergoing orthodontic

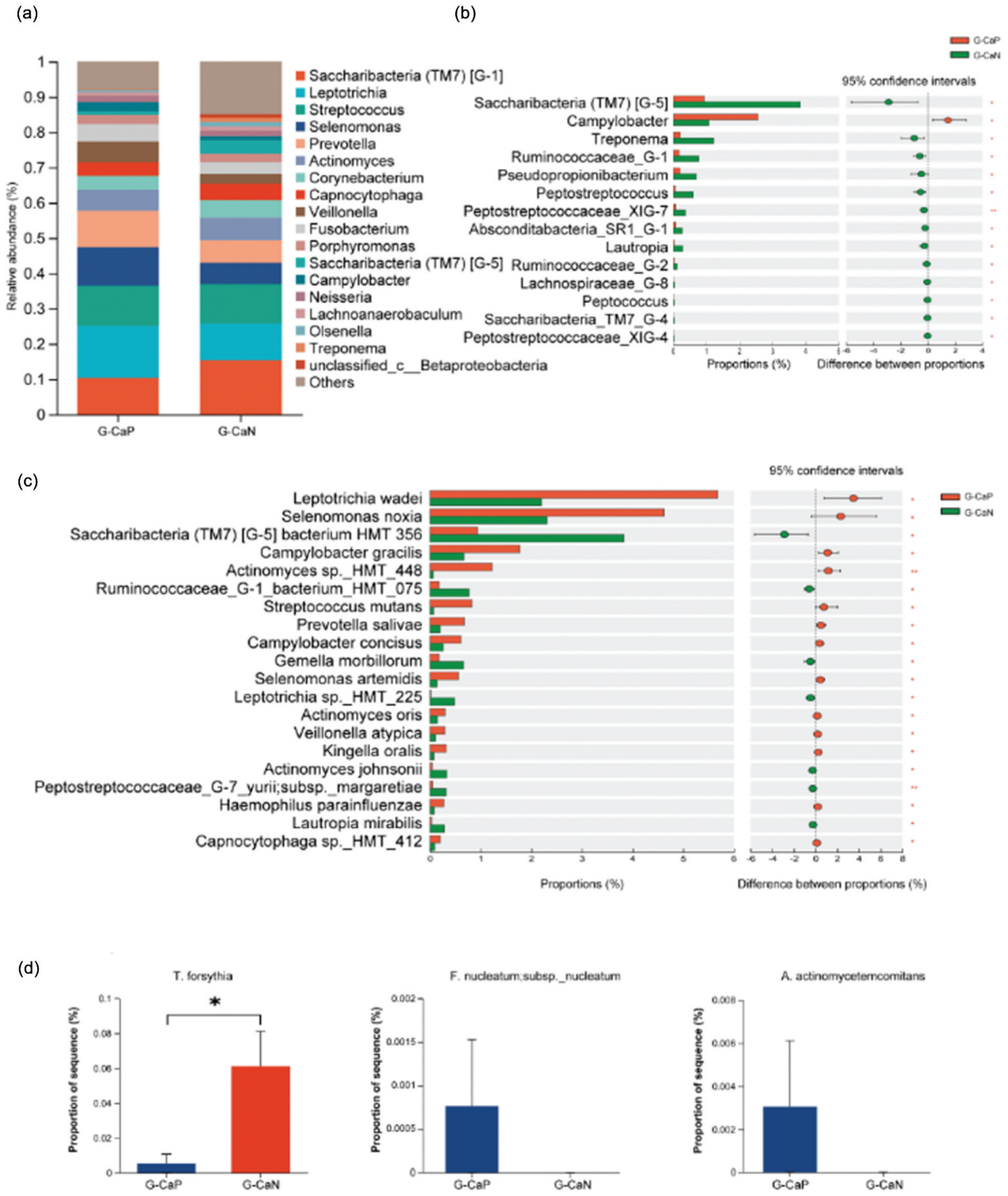

Figure 5. Species composition analysis between G-CaP and G-CaN group. (a) Microbial composition at the genus level between the G-CaP and G-CaN group. (b) The genera with significant differences between the G-CaP and G-CaN group (taking the top 1–15 bacteria ranged by relative abundance). (c) The species with significant differences between the G-CaP and G-CaN group (taking the top 1–25 bacteria ranged by relative abundance). (d) Periodontal pathogens with low relative abundance but significant differences between the G-CaP and G-CaN group (*p < 0.05, *p < 0.01, **p < 0.001).

treatment, which significantly exceeded the 15% in adolescents without such treatment. Additionally, these patients exhibited a heightened gingival bleeding index [39]. Moreover, in adolescents undergoing orthodontic treatment, factors such as poor oral hygiene, and hormonal changes contribute to a more pronounced response to dental plaque and an increased risk of periodontal hyperplasia. Studies indicate that adolescents with gingivitis are more prone to gingival bleeding and may experience attachment loss in adulthood compared to periodontal healthy adolescents [40].

High-throughput sequencing suggested that patients with gingivitis exhibited significantly greater alpha diversity in their oral microbiome than healthy individuals, along with distinct clustering characteristics. These findings correspond to previous studies showing that periodontitis patients have significantly higher microbial diversity than healthy individuals [10,41]. In this study, we found that *TM7* species *HMT-346* and *356* were enriched in patients with gingivitis, consistent with previous studies [42,43]. *Saccharibacteria (TM7)* is commonly found in human oral, skin, and gut microbiomes [44,45] and is typically dominant in inflammatory environments, particularly in periodontal-related diseases, such as periodontitis [46] and gingivitis [13]. Although *Saccharibacteria (TM7)* is generally considered a potentially pathogenic bacterium that could initiate or aggravate periodontitis and is categorized as a pathogenic red complex [47], some studies suggest it may have a protective effect by attenuating the pathogenicity of other bacteria [33]. Therefore, the precise role of *Saccharibacteria (TM7)* in gingivitis pathogenesis is yet to be elucidated. We found that *Selenomonas*, specifically *S. sputigena*, was enriched in patients with gingivitis [48]. The increased abundance of *S. sputigena* in dental plaque is closely associated with periodontal disease. *S. sputigena* adheres to gingival keratinocytes and induces the expression and secretion of cytokines and chemokines related to inflammation and leukocyte recruitment. Interaction between *S. sputigena* and the host may lead to bacteria-induced inflammation and tissue destruction, resulting in the progression of gingivitis [48]. Patients with chronic periodontitis exhibit significantly higher detection rates of *A. dentalis* than those of *A. naeslundii* or *A. oris* [49]. Our study findings support this observation, showing that *A. dentalis* is more abundant in the Gingivitis group, suggesting its potential role in altering dental plaque composition between healthy and periodontal disease states [49]. Additionally, we observed a high accumulation of *S. anginosus* and *S. oralis subsp. _tigurinus_clade_071* in patients with gingivitis, consistent with previous research [1,50]. However, further studies are required to elucidate their role in gingivitis pathogenesis.

In adolescent patients undergoing orthodontic treatment, elevated hormone levels can impact the oral microbiome and exacerbate gingival hyperplasia, leading to increased gingival sulcus depth. There is increasing evidence that periodontal tissue response is regulated by hormones such as androgen, estrogen, and progesterone. Several factors influence the incidence and severity of gingivitis in adolescents, including dental plaque biofilm, dental caries, oral respiration, and tooth crowding. Notably, steroid hormone levels are a remarkable modifier of plaque-induced gingivitis in adolescents [51]. Endocrine changes enhance the response of gingival tissue to local irritants like plaque. Despite this, dental plaque remains the primary cause of gingivitis in adolescents, and eliminating local plaque stimulation is crucial for treatment [51]. It has been reported that maintaining oral hygiene is more critical for gingival health than the increase in steroid hormone levels among adolescents [52]. Moreover, reducing gingivitis in adolescents involves removing dental plaque clinically and reducing pathogen abundance rather than regulating hormones. Our study confirmed significant differences in the marginal supragingival plaque microbiomes between adolescent patients with and without gingivitis undergoing fixed orthodontic treatment. These results indicate that while hormone levels affect periodontal tissue, the microbiomes of the Gingivitis and Periodontal healthy groups significantly differ. This study enrolled adolescents aged 11–18 undergoing fixed orthodontic treatment. The average age of the Gingivitis group (14.17 ± 1.60) was similar to that of the Periodontal healthy group (14.58 ± 1.52), suggesting comparable hormone levels, which could similarly impact the oral microbiome. However, as hormone levels vary with age, gender, and individual differences, the average age serves only as a preliminary reference, and represents a limitation of this study.

Currently, research on the relationship between hormones and gingivitis in adolescents mostly focuses on the correlation between hormones and clinical symptoms of gingivitis, with few studies examining the relationship between hormones and oral microbiomes. Previous longitudinal studies have compared pre-adolescent and adolescent orthodontic treatments to assess the impact of hormone levels on clinical and microbiological parameters. These studies reported a statistically significant increase in gingival inflammation and abundance of *P. intermedia* compared to baseline values, which may be associated with increased systemic hormone levels [53]. The results of another study indicated that the abundance of *C. rectus* was positively correlated with estradiol levels [54]. Additionally, estradiol levels in saliva were associated with the abundance of *C. gingivalis*, *Peptostreptococcus micros*, *T. denticola*, and *T. forsythia* [55]. Among these bacteria, *P. intermedia*, *T. denticola* and *T. forsythia* were relatively abundant in the Gingivitis group compared to the Periodontal healthy group, although their relative abundances were low. For the core genera and species with high abundance in this study, there is no existing literature on the hormonal effects on these microorganisms. Conversely, another study suggested that changes in pubertal hormones do not promote the

colonization of periodontitis pathogens [56]. Therefore, the relationship between hormones and periodontal pathogens warrants further investigation.

As the most detected fungus in the oral cavity [57], *C. albicans* can aggregate within subgingival biofilms of periodontitis patients, exhibiting a significantly positive correlation with periodontal disease and playing a role in the initiation and progression of periodontitis [18,58]. Previous studies have primarily focused on subgingival plaque in adults, while *C. albicans* colonization rates in the supragingival plaque of adolescents with gingivitis undergoing orthodontic treatment have not been extensively investigated. Our results suggest that a high *C. albicans* colonization occurs in the supragingival plaque of adolescent patients with gingivitis. Moreover, *C. albicans* enrichment influences the microbial composition of gingival plaques.

Co-aggregation of *C. albicans* and *A. oris* has been previously reported. These exhibit growth synergism and can form a strong dual-species biofilm, thereby increasing the total biofilm biomass [59,60]. In addition, although the relative abundance was low, we detected *F. nucleatum* and *A. actinomycetemcomitans* only in the G-CaP group. *F. nucleatum* can aggregate with *C. albicans* by binding to mannose receptors on the surface of *C. albicans* [18,61]. *A. actinomycetemcomitans* and *C. albicans* exhibit symbiosis and increased virulence, which aggravates periodontal tissue destruction [62]. Furthermore, the invasion of *C. albicans* hyphae has been detected in the gingival connective tissue of patients with periodontal disease, which is also related to the enrichment of *A. actinomycetemcomitans* [63,64]. Other bacteria enriched in the G-CaP group in this study, such as *C. concisus*, *C. gracilis*, *S. noxia*, and *V. atypica*, are presumed to be involved in the occurrence and development of periodontal disease. For example, *C. gracilis* is enriched in the plaque of patients with refractory periodontitis [65]. *C. concisus*, which belongs to the green complex, is primarily associated with the early formation of subgingival biofilms linked to early-onset periodontitis [66]. *S. noxia* can exist within supragingival or subgingival biofilms [67] and may contribute to the transition of periodontal tissues from a healthy to a diseased state [68]. *V. atypica* possesses a multivalent hemagglutinin that promotes adhesion to *P. gingivalis* and oral buccal cells [69], making it more likely to be enriched in refractory periodontitis [65]. However, further investigation is needed to understand the interactions between the bacteria and *C. albicans*.

This study confirmed that the occurrence of gingivitis during orthodontic treatment in adolescents impacts the microbial ecology of the oral microbiome, leading to changes in both the bacterial microbiota and *C. albicans*. By monitoring changes in biomarkers reflecting periodontal status, it is expected to be utilized for diagnosing and predicting gingivitis in orthodontic patients, enabling the implementation of preventive measures. However, one of the limitations of this study was the relatively small sample size, which should be increased in future research. Additionally, the interactions between *C. albicans* and various periodontal pathogens warrant further investigation to elucidate their mechanisms in the onset and progression of gingivitis. Moreover, this study is cross-sectional. Future research should involve designing a cohort study to explore the relationship between hormonal changes, clinical symptoms, and changes in bacterial and fungal abundance. This could provide a foundation for reducing the incidence of gingivitis among adolescent orthodontic patients.

Conclusions

The study identified significant differences in both microbial diversity and composition within the supragingival microbiome between adolescent orthodontic patients with gingivitis and those in a healthy state. Additionally, we observed that *C. albicans* was more prevalent in patients with gingivitis, impacting the microbiome composition of supragingival plaque in adolescent patients undergoing orthodontic treatment. This suggests a potential role for *C. albicans* in the pathogenesis of gingivitis.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Ethical approval

This study was approved by the Ethics Committee of the Beijing Stomatological Hospital (CMUSH-IRB-KJ-YJ -2022-11).

Author contributions

Hao Yang was responsible for data collection, processing and analysis, and paper writing. Hongyu Gao assisted in data acquisition, processing, and analysis. Xianju Xie provided experimental samples and participated in the research design. Hongmei Wang provided samples to assist in data processing and analysis, and Xiaowei Li provided experimental samples and method selection. Correspondence authors are Yansong Ma and Yuxing Bai. Yansong Ma was responsible for the planning and design of the whole study to ensure the integrity and accuracy of the study. Yuxing Bai designed research methods, and supervised and coordinated the research process, and provided economic and technical support.

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