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Oral microbial diversity analysis among atrophic glossitis patients and healthy individuals

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ABSTRACT

Atrophic glossitis is a common disease in oral mucosal diseases. The Current studies have found the human oral cavity contains numerous and diverse microorganisms, their composition and diversity can be changed by various oral diseases. To understand the composition and diversity of oral microbiome in atrophic glossitis is better to explore the cause and mechanism of atrophic glossitis. The salivary microbiome is comprised of indigenous oral microorganisms that are specific to each person, exhibits long-term stability. We used Illumina MiSeq high-throughput sequencing based on the V3-V4 region of the bacterial 16S rRNA gene and the internal transcribed spacer (ITS) region of fungal rRNA genes from saliva in atrophic glossitis patients and healthy individuals to explore the composition and diversity of oral microbiome. In our reports, it showed a lower diversity of bacteria and fungi in atrophic glossitis patients than in healthy individuals. The data further suggests that *Lactobacillus* and *Saccharomycetales* were potential indicators for the initiation and development of atrophic glossitis. Moreover, we also discuss the relationship between the oral microbial ecology and atrophic glossitis.

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Introduction

Atrophic glossitis is known as smooth tongue, tongues with it exhibit a smooth, glossy appearance with a red or pink background. The smooth quality is caused by the atrophy of filiform papillae. Histologically, atrophic glossitis is characterized by epithelial atrophy and varying degrees of chronic inflammation in the subepithelial connective tissue. It commonly occurs due to nutritional deficiencies such as vitamin B12, folic acid, iron deficiencies, or celiac disease [1–3]. Other aetiological factors include hyposalivation and candidiasis infection [4–6]. Currently, most studies suggested that *Candida albicans* was the primary component of the oral microbiota that was relevant to atrophic glossitis. *Candida* is the easiest genus to separate in the oral cavity and is one of the most common taxa of fungi that contribute to the early formation of in vitro biofilms [7,8]. However, apart from *Candida albicans*, little is known about other species in the oral microbiota that are correlated with atrophic glossitis. The oral microbiome, including bacteria and fungi, involved in atrophic glossitis should be explored.

Data from culture and molecular studies have collectively revealed that more than 700 species may live in the human oral cavity: approximately 600 bacterial species and 100 fungal species [9–12]. A complex role of the oral microbiome and variations in its composition are thought to be correlated with oral diseases, including dental caries, halitosis, periodontal disease, and apical periodontitis [13]. Therefore, an understanding of both oral microbial composition and diversity are crucial to evaluate their relationships with the healthy status of the host. Currently, there is no report about the oral microbial composition and diversity of atrophic glossitis, and we know only that *Candida* is most importantly related to atrophic glossitis.

Many strategies have been used to analyse the characterization and variability in the oral microbiota [14]. Culture-based methods have been traditionally used to study the diversity of the oral microbiota, but early microscopy studies have already suggested that roughly one-half of the oral microbiota cannot be cultivated in vitro [15,16]. Currently, modern techniques are used to overcome these obstacles, and molecular methods for microbial identification can be directly used in clinical samples to detect unexpected (open-ended analysis) and target-specific taxa (closed-ended analysis) in a given environment, which found that 70% were cultivable and 30% uncultivatable [13,17,18]. Oral sites, especially saliva, have the highest evenness, while buccal mucosa and keratinized gingiva have lower alpha diversity than the other oral sites [19,20]. Surprisingly, the salivary microbiome as revealed by 16S rRNA gene sequence-

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based analyses was significantly more robust [21]. At present, broad-range polymerase chain reaction (PCR) amplification of 16S rRNA followed by cloning and Sanger-based sequencing to obtain a more comprehensive profile (abundance and diversity) of the oral microbiome V3-V4 regions are considered the most reliable and capable method of providing the entire picture of bacteria[B. 22]. A more comprehensive profile of the fungal microbiome can be obtained by amplifying the universal internal transcribed spacer (ITS) region of the rRNA gene (analogous to 16S rRNA sequencing in bacteria), which has broad fungal specificity, especially at low taxonomic levels [23,24].

In our study, we characterized the oral microbiome in 50 adults who had atrophic glossitis or were healthy, amplifying the V3-V4 region of the bacterial 16S rRNA gene and the ITS region of the fungal rRNA gene in saliva to describe the community structures and diversity of the oral microbiome.

Materials and methods

Study population

The study population is consisted of 76 women and 24 men with 40 years or older from Shanxi Provincial Peoples Hospital. The oral microbiome in 50 participants with atrophic glossitis and 50 healthy participants was characterized. For the atrophic glossitis subjects, their lingual papillae on the dorsum of the tongue decreased by more than 50%, and long-term areas of damage with pain persisted more than 2 months; none used any broad-spectrum antibiotics or antifungal medicine for nearly 2 weeks. None of the patients used glucocorticoids or immunosuppressants within the 3 months, and there was no tumour recurrence. All participants provided written informed consent for all study procedures, including questionnaires and saliva sample collection. Written informed consent was obtained from the all participants and ethical approval for the study was obtained from the Ethics Committee of Shanxi Provincial People's Hospital (No-51).

Saliva sample collection

The participants were provided the Saliva DNA Sample Collection Kit (made in China) for collecting saliva according to the manufacturer's instructions. At the time of consent, each participant provided descriptive phenotypic information, including their age, sample number, type, and time. The stimulated saliva sample produced during the last 2 min (2 ml) was collected in sterile plastic tubes, where upon receipt, they were immediately stored at −80°C in freezers for DNA extraction at the time of analysis.

DNA extraction and PCR amplification

DNA was extracted from saliva samples by using the QIAamp DNA Mini kit (Qiagen, Germany) following the manufacturer's instructions. Genomic DNA quality was detected by a Thermo NanoDrop 2000 UV micro-ultraviolet spectrophotometer (Thermo Fisher Scientific, USA) and 1% agarose gel electrophoresis. Bacteria and fungi presenting these samples were identified with 16S probes and ITS-based probes, respectively. For bacterial identification, the V3-V4 region of 16S rRNA was amplified in triplicate using primers with the universal primer set (forward primer 341 F:5′ CCTACGGGNGGCWGCAG 3′; reverse primer 805 R: 5′ GACTACHVGGGTATCTAATCC 3′). For fungi, the ITS region from rRNA sample extracts was amplified in triplicate using fluorescently labelled forward primer ITS3 (5′ GCATCGATGAAGAACGCAGC 3′) and unlabelled reverse primer ITS4 (5′ TCCTCCGCTTATTGATATGC 3′). PCR amplification was carried out using a TopTap DNA polymerase kit (Transgen, China) under the following conditions: 94°C for 2 min; followed by 25 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min; and a final extension at 72°C for 10 min. PCR amplification products were purified using an Agencourt AMPure XP Kit (Beckman Coulter, Brea, CA, USA) according to the manufacturer's instructions. The DNA products were pooled together with Agencourt AMPure XP PCR Purification Beads (Beckman Coulter, USA) at equal volumes for purification and then examined by 1% agarose gel electrophoresis to obtain the original library. The library was diluted properly. Quantification was performed using an Invitrogen Qubit3.0 Spectrophotometer (Thermo Fisher Scientific, USA), and samples were pooled together with corresponding proportions of DNA according to the sequencing throughput requirements of different samples. Library quality and fragment size were checked by an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The quantified and homogenized PCR products were sequenced on an Illumina MiSeq PE250 instrument (Illumina, USA).

Sequence read processing and OTU cluster analysis

Multiplexed paired-end sequences shorter than 20 bases and adaptor sequences were removed by using TrimGalore software to obtain long reads for hypervariable regions and exclude poor-quality reads. FLASH2 software [25] was used to assemble the paired sequences to obtain a merged sequence. Mothur software was used to find and remove primers in the sequence. Sequences with a total base

error rate greater than 2 reads in total bases and length less than 100 bp were removed by Usearch software to obtain clean reads. UPARSE software was used to discard singleton sequences. Sequences with similarity >97% were aggregated into the same operational taxonomic units (OTUs). An OTU table of raw counts was normalized to form an OTU table of relative abundance values. The same types of taxa were agglomerated at the phylum, class, order, family and genus levels. The resulting OTU table was used for subsequent species annotation and subsequent statistical analysis.

Bioinformatics analysis

Species abundance analysis uses visual analysis methods to show the species composition in each sample at a certain classification level. The species composition was plotted in a bar chart and displayed on a graph, which presents the composition, relative abundance and proportion of species in each sample and two groups more intuitively.

A heatmap diagram [26] based on distance was used to cluster species of samples. It further displayed their relative abundance changes transformed by Z-score in the groups and reflected the similarities and differences in composition of species in all samples at a specific classification level [27].

Alpha diversity analysis measured significant differences in oral microbial diversity and richness between the two groups. The most well-known and accepted alpha diversity indices were used to evaluate the diversity and richness of biological communities, including the Shannon and Chao1 indices respectively. The diversity and richness of samples for bacteria and fungi were compared from the atrophic glossitis and control groups by the Wilcoxon rank-sum test. The Chao1 and Shannon indices were calculated at 97% identity by mothur software [17].

Beta diversity analysis is often characterized using the number of species shared between two communities. Principal coordinates analysis (PCoA) based on the data matrix using a Bray Curtis distance metric, which exhibited diversity within samples and similarity between samples. However, the significant differences of species between the atrophic glossitis group and control group were not observed in PCoA. ADONIS analysis [28] was used to display the significant differences of species among groups based on Bray-Curtis distances by permutational multivariate analysis of variance. It was used to test for significantly differences between two groups at the P-value<0.05 level.

LEfSe was used to determine taxon differences. The enriched taxon differences between atrophic glossitis and control samples were defined by LEfSe analysis, which identified specific biomarkers discriminating the two study groups using relative abundances. The Cladogram of effect size (LEfSe) showed the taxonomic representation of significant differences between atrophic glossitis group and control group. The linear discriminant analysis (LDA) of effect size (LEfSe) supports high-dimensional classification comparison and was performed to determine the differently enriched taxa between two groups. The threshold for the logarithmic LDA score for distinguishing features was set to 2.0 (p-value<0.05) [29].

Statistical analysis

For taxon assignments and measurements, forward and reverse sequences from the FASTQ files were analysed separately using the software package QIIME2.9. The reads identified in closed reference picking from the Ribosomal Database Project (RDP; http://rdp.cme.msu.edu/index.jsp) were used for the follow-up analysis. All diversity analysis were conducted using R (version 3.0.1). The Wilcoxon rank-sum test was used to compare the diversity and abundance between two groups, with p-value<0.05 considered a significant difference.

Result

Participant demographics

A total of 100 individuals were enrolled in the study, with 50 atrophic glossitis patients and 50 healthy individuals (24 males and 76 females). The mean age was 65.1 ± 10.4 years in the atrophic glossitis group (age range: 45–85) and 60.9 ± 11.6 years in the control group (age range: 41–89). Participants with atrophic glossitis did not differ significantly from control participants in age and sex distribution (all p-values > 0.05). The demographic and clinical characteristics of the subjects are displayed in (Table 1). Cigarette, Alcohol, Oral health status, Coronary disease (CAD), and Parageusia did not differ from atrophic glossitis group with control group (all p-values>0.05). Diabetes mellitus (DM), Hypertension, Degree of atrophic, Anemia, Wetness of tongue body, and Glossodynia were significantly different between the atrophic glossitis group and the control group (all p-values <0.05).

Microbial community structure

Our results showed that about main bacterial and fungal genera with relative abundance >1% in the oral microbiota of the study changed at the genus level Figure 1(a,b). For bacteria, *Streptococcus, Prevotella, Neisseria, Rothia, Veillonella, Haemophilus, Fusobacterium* and *Leptotrichia* were the most common genera in the bar plot. For fungi,

Table 1 Clinical characteristics of atrophic glossitis patients and healthy individuals.

Characteristic	atrophic glossitis group(n = 50)	control group (n = 50)	P-value
Age mean±SD	65.1 ± 10.4	60.9 ± 11.6	0.006
Sex, n (%)			0.349
Male	10(20)	14(28)	
Female	40(80)	36(72)	
Cigarette, n (%)			0.120
Yes	2 (4)	3 (6)	
No	48 (96)	47(94)	
Alcohol, n (%)			0.359
Yes	1 (20)	4 (8)	
No	49 (98)	46 (92)	
Oral health status, n (%)			0.878
Good	1 (2)	2 (4)	
General	29 (58)	27(54)	
Poor	20 (40)	21(42)	
DM, n (%)			0.006
Yes	8(16)	0 (0)	
No	42(84)	50 (100)	
CAD, n (%)			0.207
Yes	5 (10)	0 (0)	
No	45 (90)	49 (98)	
Hypertension, n (%)			0.002
Yes	15 (30)	3 (6)	
No	35 (70)	47 (94)	
Anemia, n (%)			0.036
Yes	8 (16)	1 (2)	
No	42 (84)	49 (98)	
Parageusia, n (%)			0.318
Have	6 (12)	3 (6)	
lost	1 (2)	0 (0)	
No	43 (86)	47 (94)	
Wetness of tongue body, n (%)			0.007
Slight	15 (30)	12 (24)	
Moderate	11 (22)	3 (6)	
Severe	3 (6)	0 (0)	
No	21 (42)	35 (70)	
Glossodynia, n (%)			0.000
Yes	34 (68)	11 (22)	
No	16 (32)	39 (78)	
Degree of atrophy, n (%)			0.000
Slight	16 (32)	1 (2)	
Moderate	21 (42)	0 (0)	
Severe	13 (26)	0 (0)	
No dry	0 (0)	49 (98)	

Saccharomycetales_unidentified_1, *Agaricomycetes_unidentified_1*, *Saccharomyces*, *Fungi_unidentified_1_1*, *Aspergillus*, *Candida*, and *Malassezia* were the most common genera.

Comparing the two participant groups allows the relative abundance of bacteria and fungi to be visualized Figure 2(a,b). For bacteria, *Streptococcus*, *Rothia*, *Porphyromonas* and *Gemella* were more relative abundant in the atrophic glossitis group than in the control; *Prevotella*, *Neisseria*, *Veillonella*, *Fusobacterium*, and *Leptotrichia* were less relative abundant in the atrophic glossitis group than in the control. For fungi, *Saccharomycetales_unidentified_1*, *Candida* was more relative abundant in the atrophic glossitis group than in the control; *Agaricomycetes_unidentified_1*, *Saccharomyces*, *Aspergillus*, and *Malassezia* were less relative abundant in the atrophic glossitis group than in the control.

The main bacterial and fungal genera with relative abundance >1% at the genus level can be visualized in (Figure 3) where their relative abundance have Z-score values with color patterns. The discriminating species as well as the samples (atrophic glossitis group and control group) were arranged along the two dimensions (axes) based on hierarchical clustering. Notable patterns emerged when the distributions of taxa within distinct clusters were compared between the atrophic glossitis and control groups.

The consistency of the predominant genera among groups and their overall similarity in relative abundance can be seen intuitively in visual analysis in Figure 2 (a,b), but the significant difference of relative abundances among groups cannot be compared. We further screened the following species that differed significantly between groups by the Wilcoxon rank-sum test, including 19 bacterial genera at genus level (p-value<0.05) in total (*Alloprevotella*, *Oribacterium*, *Solobacterium*, *Eubacterium*, *Sacchar ibacteria_genera_incertae_sedis*, *Lachnoanaerobaculum*, *Gemella*, *Actinomyces*, *Lactobacillus*, *Prevotella*, *Stomatobaculum*, *SR1_genera_incertae_sedis*, *Veillonella*, *Rothia*, *Treponema*, *Peptostreptococcus*, *Catonella*, *Atopobium*, *Leptotrichia*) (Figure 4a) and 1 fungal genera (p-value<0.05) (*Saccharomycetales_unidentified_1*) (Figure 4b).

Our results showed the relationship between the degree of atrophic and oral microbial communities, including bacteria and fungi Figure 8 (a,b). The atrophic degree of glossitis patients was divided into three groups (slight, moderate, severe). For bacteria, our analysis showed that *Eikenella* have the correlation with the atrophic degree in atrophic glossitis patients. There was a decrease in relative abundance with the increasing of atrophic degree and a significant difference among groups respectively (slight VS moderate; slight VS severe) (Figure 8a). For fungi, the result showed that *Saccharomycetales_unidentified_1* has no correlation with the atrophic degree and its relative abundance has no change with the increasing of atrophic degree (Figure 8b).

Alpha and beta diversity analysis

An overview of biological alpha diversity indices calculated for the investigated atrophic glossitis and control groups are shown in (Figure 5). We measured the Shannon indices and Chao1 indices of oral microbiome. The results from comparing samples of saliva were significantly different in terms of diversity between pair groups, with all samples collected from the atrophic glossitis group having slighter diversity than those from the control group, whether for

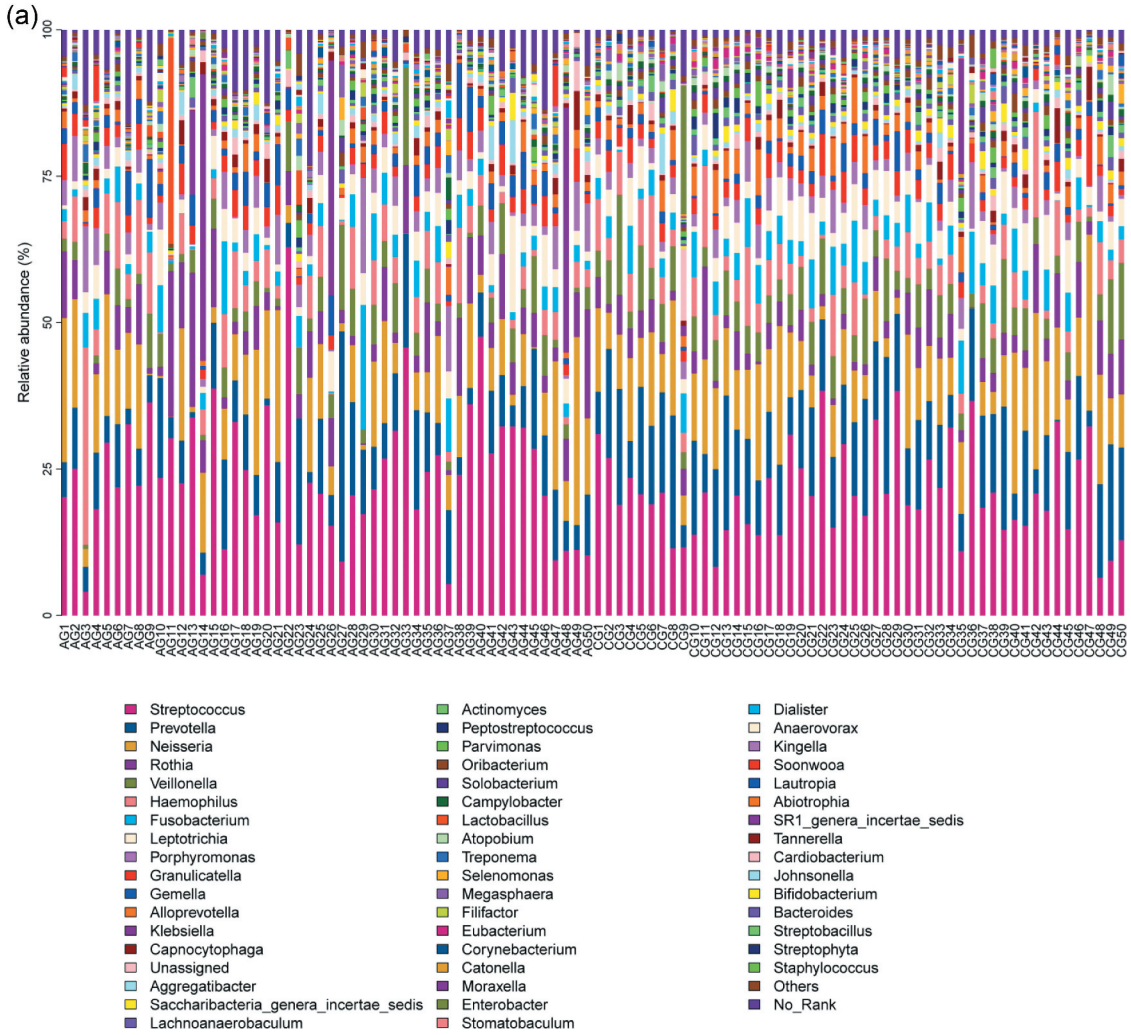

Figure 1. Overall distribution and relative abundance (>1%) of the predominant bacteria at the genus level in oral rinse samples obtained from all participants. (**a**) Bacteria (**b**) Fungi.

bacteria (p-values = 0.001899; Shannon's indices) or fungi (p-value = 0.010085; Shannon's indices). However, we found no statistically significant difference in richness between the two groups, whether for bacteria or fungi (p-value >0.05; Chao1 indices).

Beta diversity was revealed in plots from PCoA showed the similarity between pairs of samples and the relationships of individual samples to each other in 2-dimensional space. ADONIS analysis found that there was statistical significant difference between the two groups, whether for bacteria or fungi (p-value = 1.00e-04) (Figure 6).

The Cladograms of LEfSe representing the potential biomarkers of different groups Figure 7 (a,c). In the atrophic glossitis group, the most enriched bacteria were mainly *g_Gemella, g_Lactobacillus* and *g_Allisonella* at the genus level. The most enriched fungi was *f__Saccharomycetales_fam_Incertae_sedis* at the family level. In the control group, the most enriched bacteria were *g_Actinomyces, g_Mobiluncus, g_Alloprevotella, g_Prevotella, g_Eubacterium, g_Butyrivibrio, g_Lachnoanaerobaculum, g_Oribacterium, g_Stomatobaculum, g_Solobacterium*, and *g_Megasphaera* at the genus level. The most enriched fungi were *f_Sporidiobolaceae* and *f_Pichiaceae* at the family level.

Linear discriminant analysis (LDA) show significant differences abundant features represented by LDA score among groups Figure 7(b,d). In the atrophic glossitis group, the significant bacterial differences were detected, with *g_Gemella* and *g_Lactobacillus* exhibited relatively higher abundances at genus level (LDA>2, p-value<0.05); The significant fungal differences were also detected, with *f_Saccharomycetales_fam_Incertae_sedis* exhibited relatively higher abundances at family level (LDA>2, p-value<0.05); with *g_Candida* exhibited relatively higher abundances at genus level (LDA>2, p-value<0.05). In the control group, the significant bacterial differences were detected, with *g_Streptococcus.s_uncultured_organism* exhibited relatively higher abundances (LDA>2, p-value<0.05). The significant fungal differences were also detected, with *f_Sporidiobolacea* and *f_Pichiaceae* exhibited relatively higher abundances at family level (LDA > 2, p-value<0.05); with *g_Ogataea* exhibited relatively

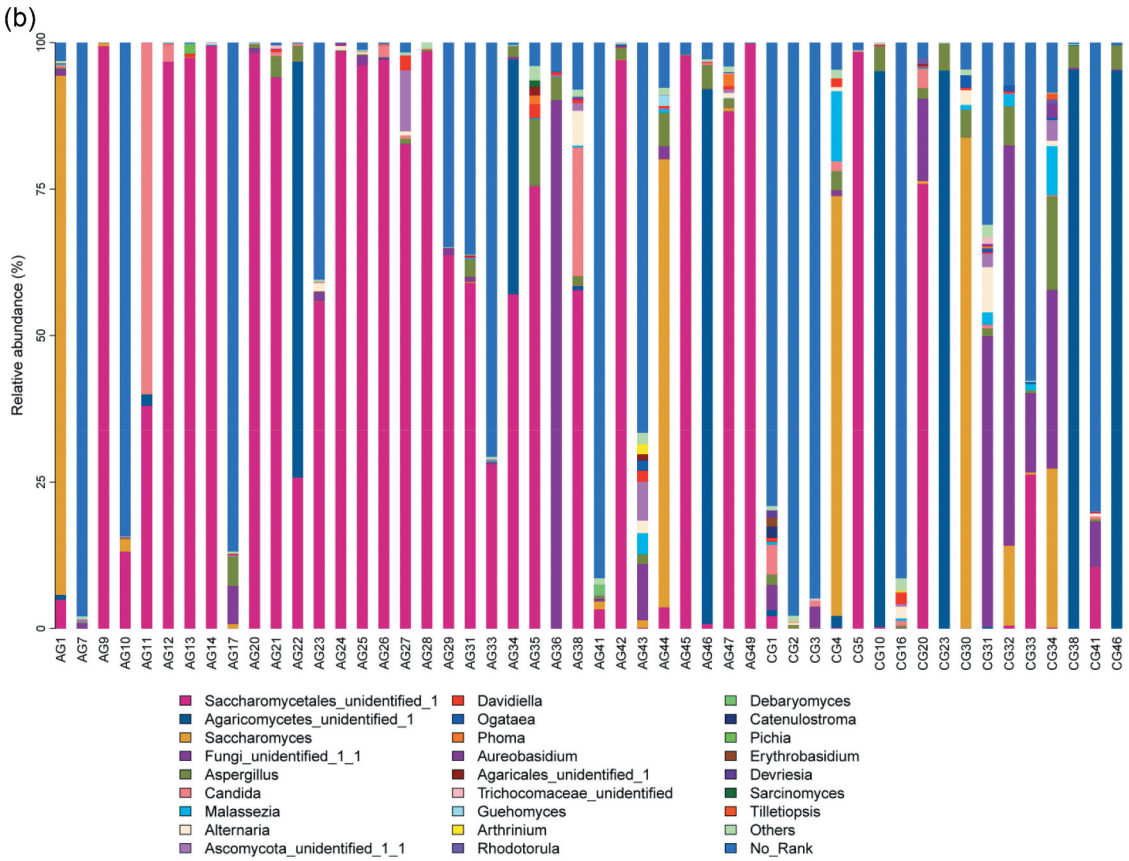

Figure 1.(continued)

higher abundances at genus level (LDA > 2, p-value<0.05).

Discussion

Atrophic glossitis has been a significant oral health issue in humans associated with several conditions, not directly attributable to mechanical damages of the mucosa, can occur in systemic or local conditions [30]. With the ageing of the population, the elderly have increasingly suffered from atrophic glossitis in recent years. In previous studies, *Candida albicans* has been reported to have a higher prevalence in patients with atrophic glossitis, and it is essential in the initiation and progression of atrophic glossitis [6–8,31]. The oral cavity is home that hold one of the most complex microbial communities, comprising mainly bacteria, viruses, fungi, protozoa, archaea, phages and candidate phyla radiation (CPR), which has remained understudied, but several recent reports have illustrated that oral microbial diversity is important for oral health [13,32,33]. The interactions and functions within a complex oral microbiome have become the focus of interest. Recent studies have analysed the oral microbial diversity in caries, periodontal disease, HIV, and irritable bowel syndrome and explored their relationship with the above diseases [34–37]. Therefore, a comprehensive oral microbial study is needed. It is necessary to better understand the relationship between aetiology and diseases. However, an understanding about the relationship between shifts in the oral microbiota and atrophic glossitis pathogenesis are not fully cleared, as the microbial composition and diversity are still not fully known. Here, we searched for describing bacteria and fungi simultaneously in oral atrophic glossitis patients and health care of hosts, but found none in other study.

The diabetes and hypertension were significantly different between the atrophic glossitis group and control group (Table 1). A previous study showed that diabetic patients appeared an atrophic lesion of central tongue papillary, and there was a significant association with *Candida* [38,39]. It was generally acknowledged that patients with type I diabetes mellitus were more susceptible to fungal infections, particularly to *Candida albicans* [40]. The study found that the presence of *Candida* pseudohyphae was related to elevated glycosylated hemoglobin; Hyperglycemia could contribute to the risk of *Candida* by increasing salivary glucose levels, which may promote overgrowth by *Candida* [41]. However, other study found that the colonization of *Candida* have no significant difference between diabetic patients and healthy individuals, whether type I or II diabetic patients with atrophic glossitis were not related to *Candida* infection necessarily [42].

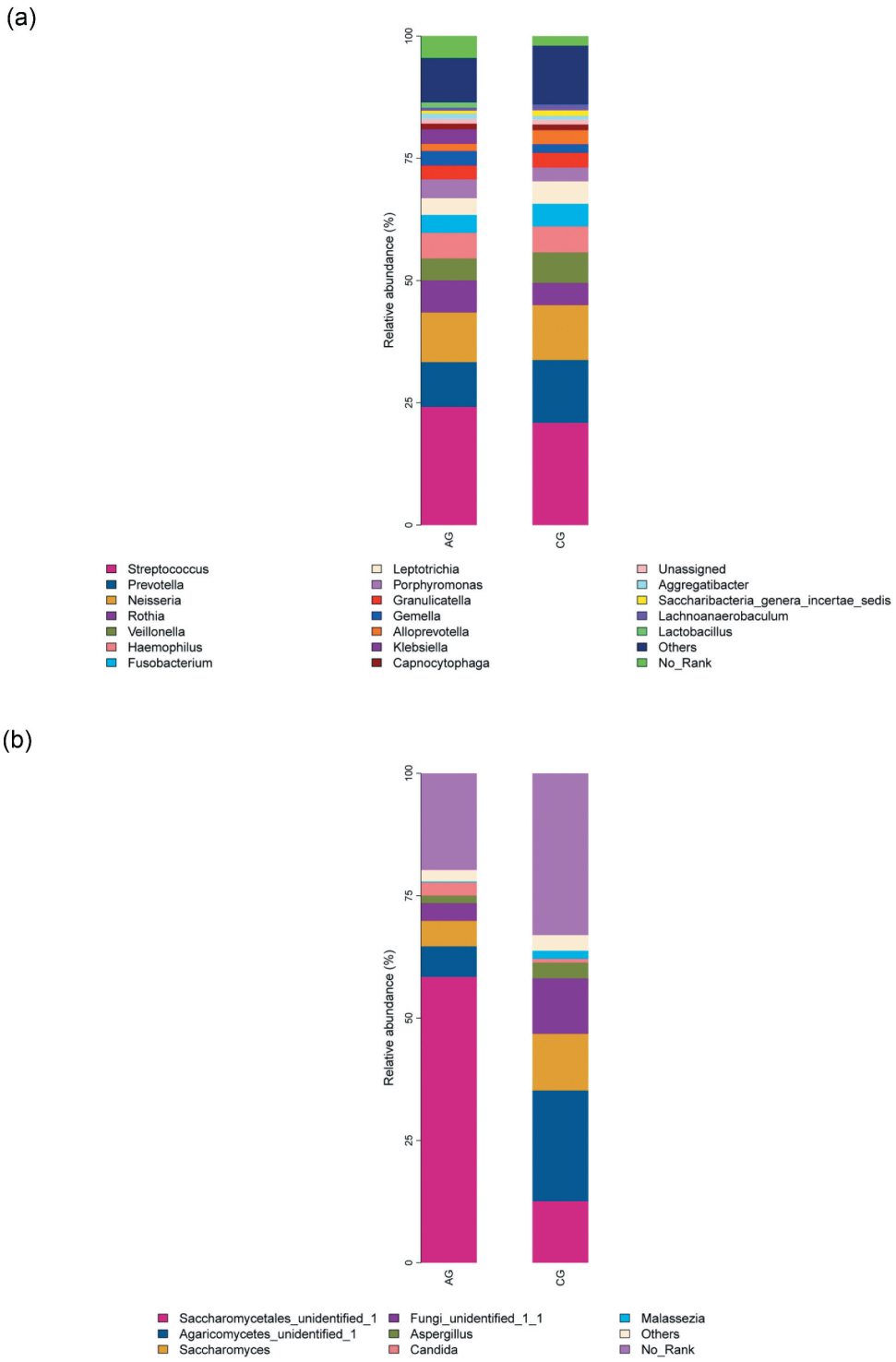

Figure 2. Genus level relative richness comparison of two groups. Mean taxonomic profiles for the main microbiome abundant genera in saliva from the atrophic glossitis group and the control group subjects. (**a**) Bacteria (**b**) Fungi.

Therefore, diabetes may also cause the occurrence or enhance the incidence of atrophic glossitis, but the view of resulting in changes of *Candida* is still controversial. In addition, about the relationship of hypertension and atrophic glossitis, there is a few research to study currently. One study found that some of patients with atrophic lesions who were taking hypotensive drugs have higher prevalence of central papillary atrophy [38]. Thus, hypertension maybe confounded variables in our study.

Our study was a pilot report on salivary microbial diversity in atrophic glossitis patients and healthy individuals. In this study, bacteria were found more, but genera of fungi were few at genus level Figure 1(a, b). We could see these species were clustered and a characteristic pattern for some of the species which assessed their relative abundance changes among groups (Figure 3). The reason for this phenomenon was mainly the relative rareness of fungi (<0.1% of the microbiome, based on cfu); genetic

Figure 3. Heatmap shows the relative abundance (>1%) of species isolates (columns) in all samples (rows) and species are clustered accordingly at the genus level, normalized and Z-score transformed. Color gradient from blue to red indicates species abundance from small to large. (**a**) Bacteria (**b**) Fungi.

material from fungi can be difficult to isolate, and many fungal species are uncultivable using current methods [4]. However, bacteria are not the only factor causing changes in oral ecology; fungi is also important, which is consistent with significant proportions of the oral microbiota in recent years [43]. Recently, bacterial-fungal ecological interactions have attracted researchers' attention. Jonathon L. Baker. et al. suggested that fungi are numerically underrepresented, but the larger cell size of fungal species creates a structural 'skeleton' for fungal–bacterial multispecies biofilms. Fungal species stimulate the host immune system in a distinct manner with disparate immunological outcomes compared to those of their bacterial neighbours, which may cause systemic inflammatory disorder [32,44]. Atrophic glossitis has multiple causes, and it is generally accepted that the clinical symptoms of atrophic glossitis are a manifestation of chronic inflammation. Therefore, the interactions in bacterial-fungal multispecies biofilms may potentially affect the oral microbial ecology of atrophic glossitis patients.

Malassezia was almost absent from the atrophic glossitis group but was found in the control group Figure 2 (a,b). A recent study of the oral fungal mycobiome of saliva samples from 15 periodontal adults found a lower frequency (17%) of *Malassezia* species [37]. Hajishengallis et al. suggested that specific low-abundance pathogens can be seen as 'keystone pathogens' that influence periodontal disease by altering the 'healthy' microflora into a disease state [45]. Can low-abundance *Malassezia* influence the progression of atrophic glossitis? We need more research to understand this issue. However, in our previous study, *Malassezia* species were high-abundance fungi in the fevers among the elderly

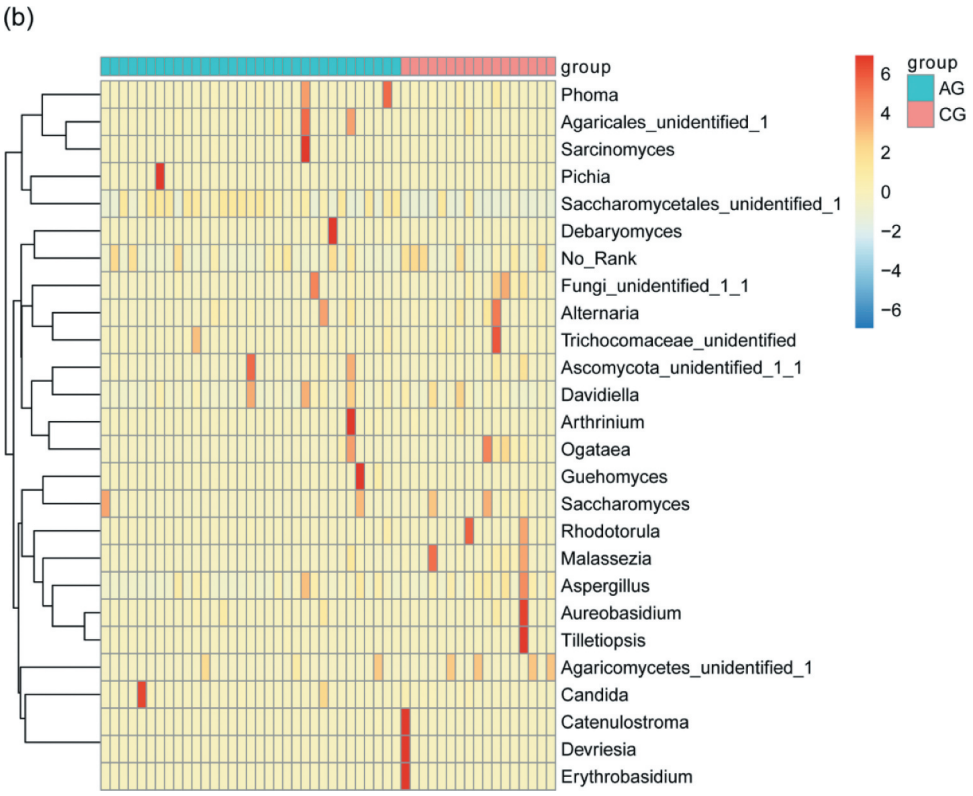

Figure 3.(continued)

and it is an important genus of fungi that be worthy for attention [46]. In addition, Dupuy et al. observed a high prevalence and abundance of the genus *Malassezia* from saliva, and include it to the core oral mycobiome; the role(s) that *Malassezia* species may play in oral health and disease, or in the dynamics of oral microbial communities, but remains to be determined [47].At the same time, researchers have proposed that the variety of microbiomes should be better investigated based on age and these differences along with different diseases [35]. Different oral diseases and age stages of patients may lead to differences in abundance and diversity in the same special oral microbiome. Perhaps this is the reason why few *Malassezia* species emerged in the atrophic glossitis group but it may have been the opposite for other oral diseases. Furthermore, the result of the high relative abundant fungal microbiome at the genus level in the control (Figure 2b) were consistent with the results of a previous report: *Candida, Saccharomycetales* and *Aspergillus* were the most abundant, which isolated from 20 healthy participants [10].

For a long time, acidogenic species of the genus *Streptococcus* have been considered the causative agent of dental caries which is highly active in caries progression [48,49]. In our study, we found that *Streptococcus* was the most abundant genus and its abundance was higher in the atrophic glossitis group than in the control group (Figure 2a), but there was no significant difference in its relative abundances between the two groups (Figure 4a). The result of LEfSe analysis was that *g_Candida* was the main genus in the atrophic glossitis group (Figure 7d). Jonathon L. Baker. et al. suggested that the capability of interspecies interactions between *C. albicans* and *Streptococcus* to exacerbate the severity of oral candidiasis, highlight the importance of interkingdom
interactions in the pathogenesis of what are increasingly recognized as polymicrobial disease [32]. Does it affect the progression of atrophic glossitis with the colonization *C. albican*? The interactions of *C. albicans* and *Streptococcus* could be as new idea to explore the pathogenesis of atrophic glossitis.

In our study, *g_prevotella* was the high relative abundance species in the atrophic glossitis and significant difference between two groups (Figure 2a, Figure 4a). In many studies, *Prevotella* was a commonly observed member of the oral microbiome with considerable variation in its relative abundance [20,50]. It has been identified as a potential pathogen in the oral cavity, where it is associated with both carious lesions[51] and periodontitis [52,53].The high *Prevotella* level could be related to local or systemic inflammatory disoders [54]. Maybe it can cause inflammation of local tongue papilla of atrophic glossitis. However, a finding in our LEfSe analysis was that *Prevotella* was not the main genus in the atrophic glossitis group but in the control Figure 7 (a,b).

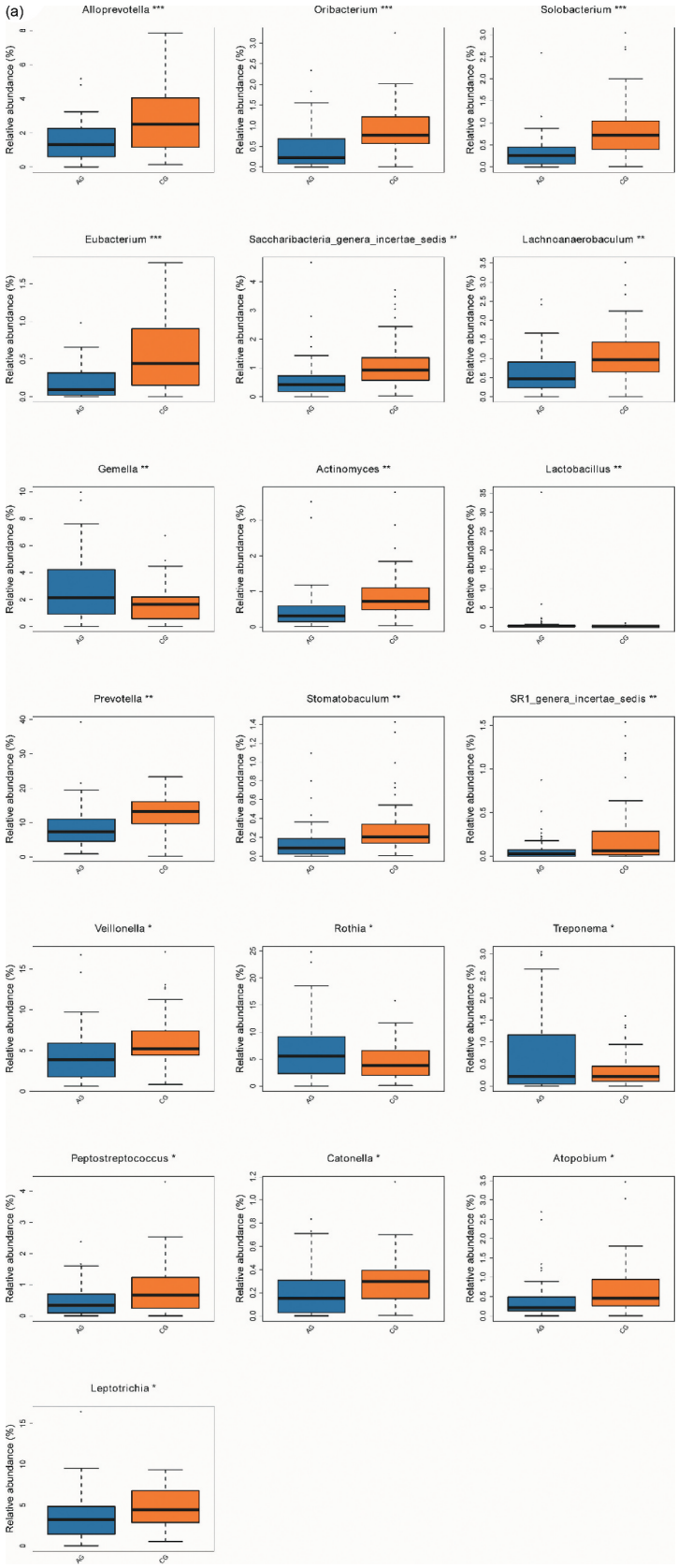

Figure 4. The significant differences species with a box diagram at the genus level among the sample groups, showing species degree of dispersion within the group and their relative abundance between different groups. (**a**) Bacteria: the box-plot shows 19 significantly different bacterial species. (**b**) Fungi: the box-plot shows 1 significantly different fungal species.

Significantly different groups are indicated with asterisk*. One asterisk (*) indicates $p < 0.05$; two asterisks (**) indicate $p < 0.01$; three asterisks (***) indicate $p < 0.001$.

It seems that there are no associations between *Prevotella* and atrophic glossitis. This may be attributed to the nonclinical nature of this study and the limatations of sample size.

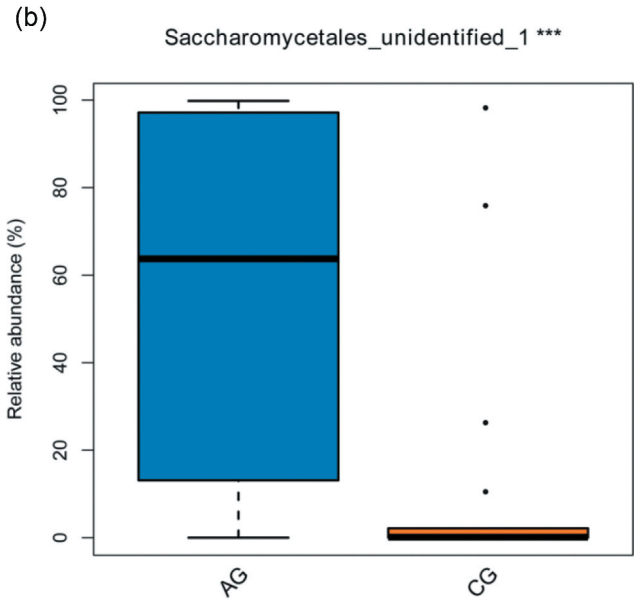

Figure 4.(continued)

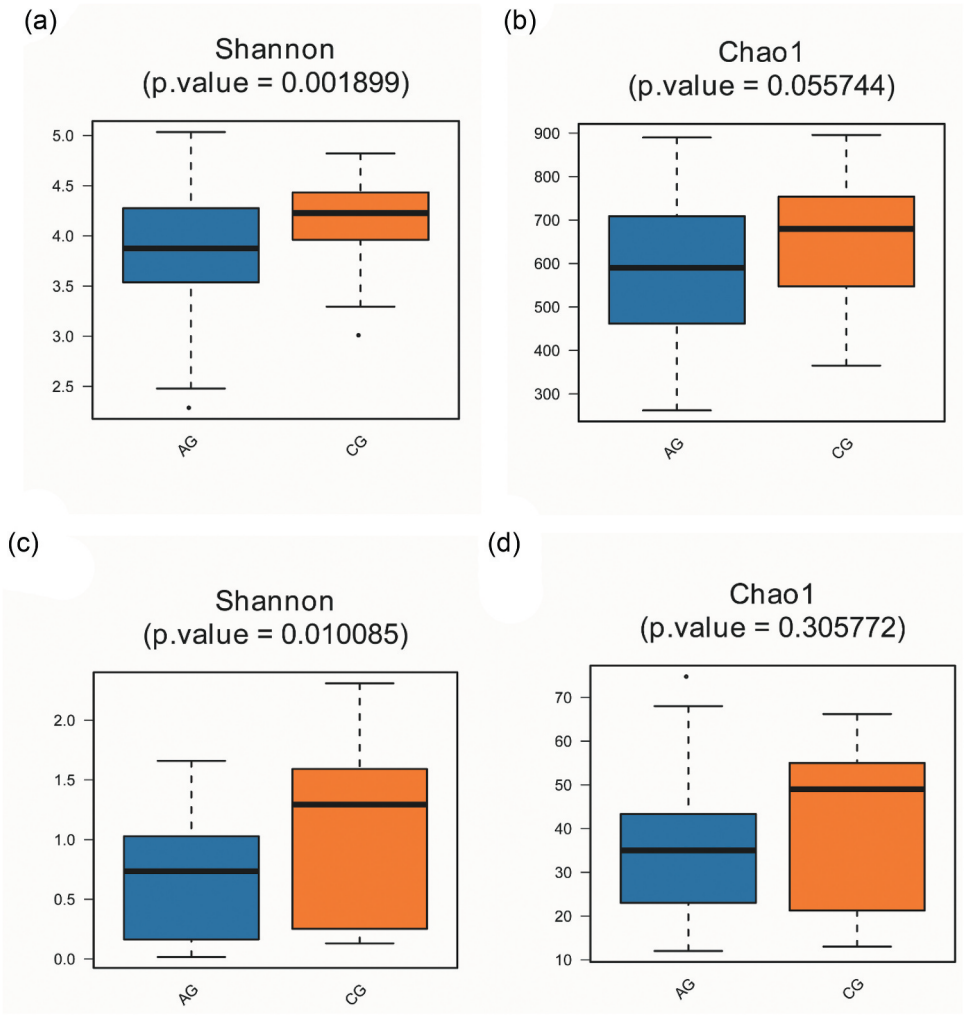

Figure 5.Alpha diversity comparisons in saliva from the atrophic glossitis group and the control group are shown. The Shannon indices revealed lower microbial diversity for saliva in the atrophic glossitis group than the control group. (**a**) Bacteria: $p = 0.001899$ (**c**) Fungi: $p = 0.010085$. The Chao1 indices revealed no difference in richness among two groups. (**b**) Bacteria: $p > 0.05$ (**d**) Fungi: $p > 0.05$.

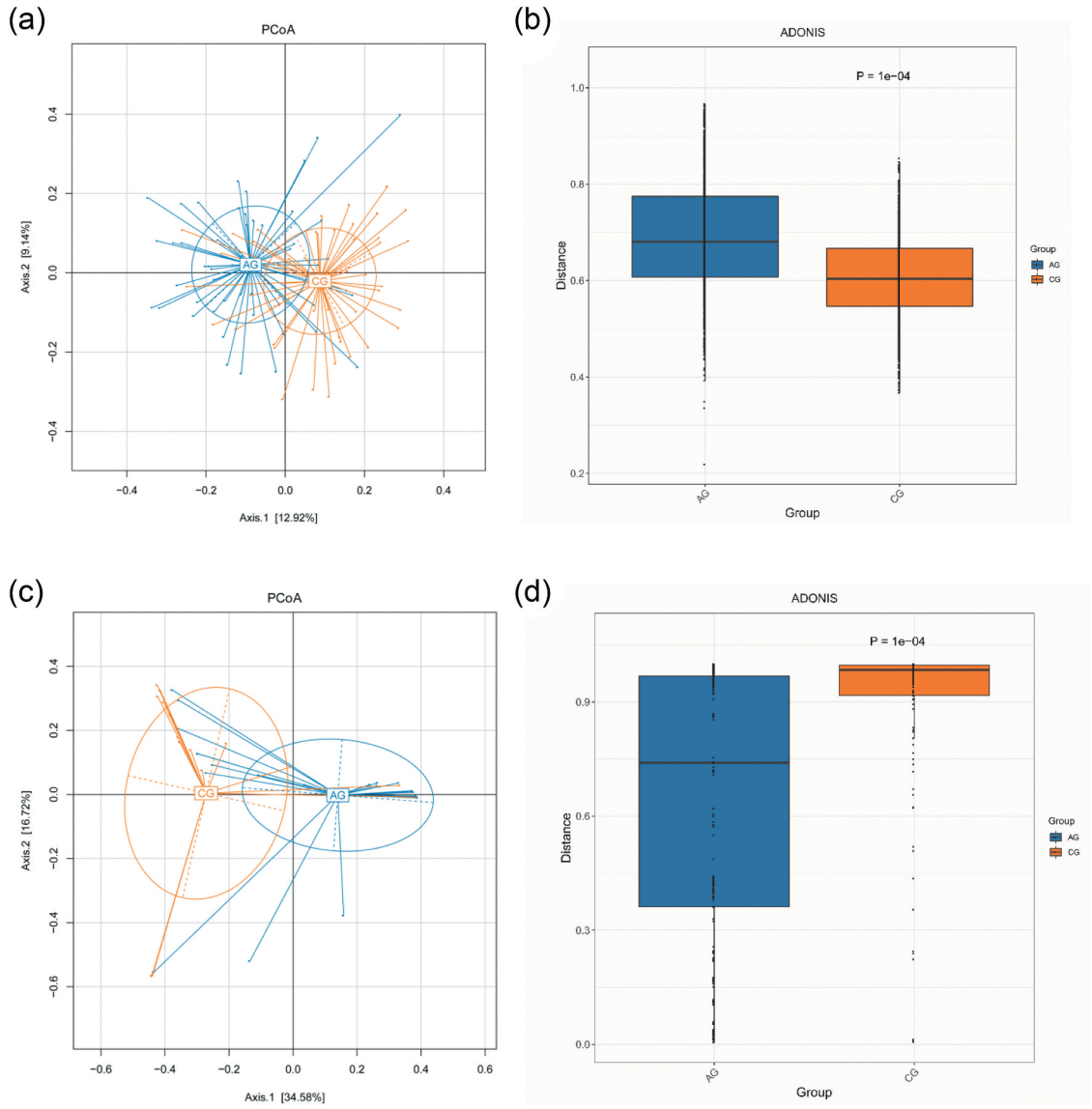

Figure 6. Beta diversity comparisons in the saliva from the atrophic glossitis group and the control group. PCoA of Bray Curtis distance express similarity or difference among two groups and within a group. Each sample is represented by a dot. Visualizing the Bray Curtis distances between samples in a scatterplot where points (representing samples) that are more distant from one another are dissimilar. The approximate proportion (%) of variance explained by each principal coordinate axis is reported in the axis label. (**a**) Bacteria: Axis1 explained 12.92% of the variation observed, Axis 2 explained 9.14% of the variation. (**c**) Fungi: Axis1 explained 34.58% of the variation observed, Axis 2 explained 16.72% of the variation. ADONIS tests further reveal that significant differences exist between two groups. (**b**) Bacteria: p = 1.00e-04. (**d**) Fungi: p = 1.00e-04.

In this study, based on the alpha diversity indices, the result demonstrated that the diversity of bacteria and fungi in atrophic glossitis patients was lower than that in healthy subjects Figure 5 (a,c). This could imply a higher degree of conformity of the microbial compositions along with physical health conditions, and mean that the oral ecology of the atrophic glossitis group may lead to a decrease in bacterial-fungal microbiome diversity. The inflammatory state of the oral mucosa may affect the changes in microorganisms in the atrophic glossitis group. Atrophic glossitis, an inflammatory disease, might influence bacterial-fungal composition and diversity. In contrast, the decrease in oral microbiome may change their diversity and cause inflammation in atrophic glossitis. Devine, D. A. et al. suggested that both inflammatory and anti-inflammatory responses may be induced in host tissues by members of the oral bacterial microbiota [55]. All the above indicated that bacterial-fungal diversity had more complex and interconnected relationships with atrophic glossitis.

For bacteria, our work found that *g_ Lactobacillus* was the low relative abundance species at the genus in the atrophic glossitis (Figure 2a), but significant difference between two groups (Figure 4a). It was the main genus in the atrophic glossitis group Figure 7 (a, b). As we all know, *Lactobacillus* species were present at significantly higher levels in caries, and their acid-generating action caused the oral environment to

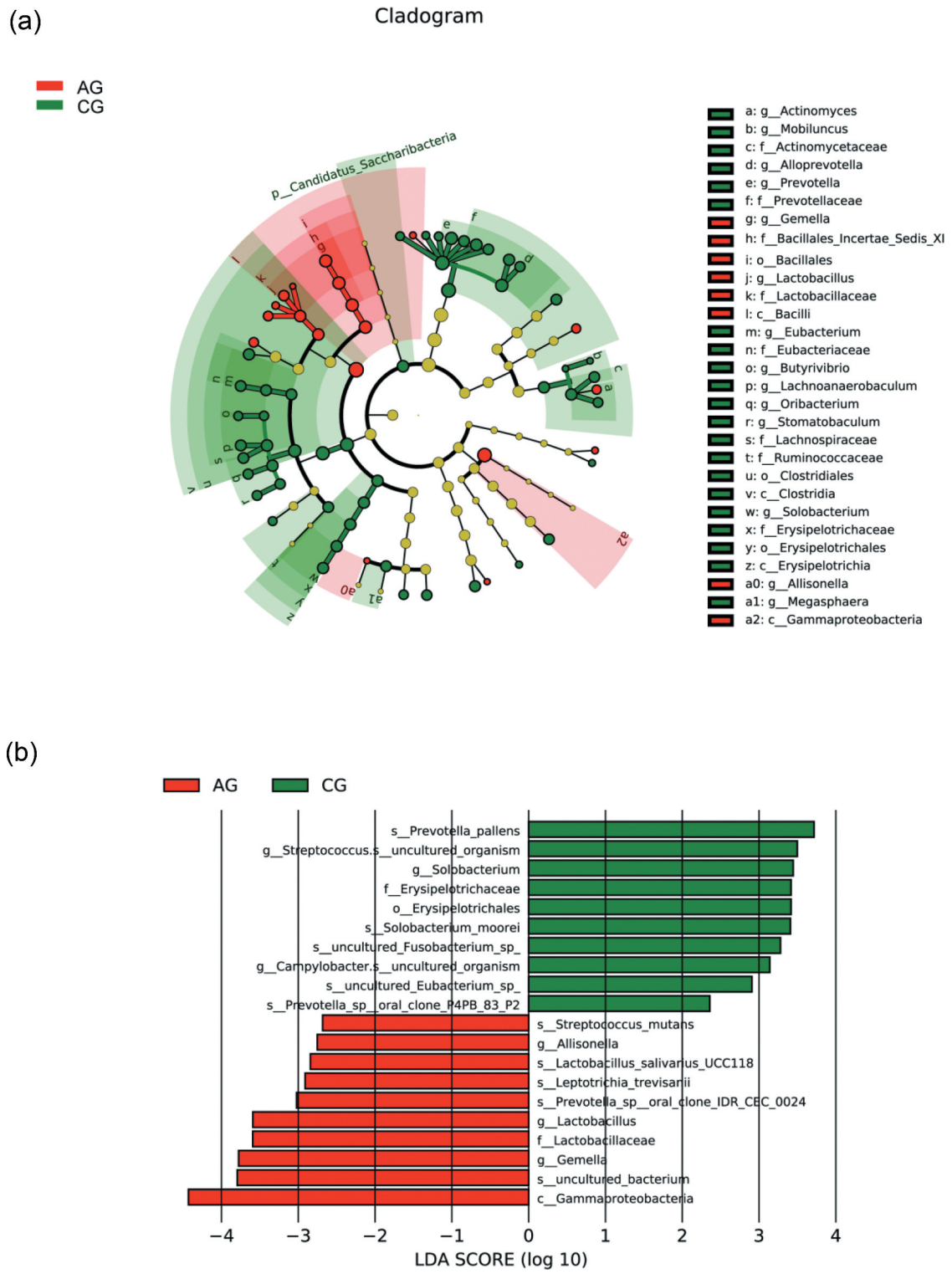

Figure 7. Biomarker analysis defined by LEfSe. Cladogram shows taxonomic representation of significant differences in relative abundance between the atrophic glossitis group and the control group. The significantly different taxa are signified by different color nodes between the atrophic glossitis group (red) and the control group (green). The diameter of the node is proportional to the relative abundance. The colored nodes from the inner to the outer circles represent taxa from the phylum to the genus level. Histogram of LDA scores show significant differences of abundant features among groups. (**ab**)Bacteria (**cd**) Fungi.

form a low-pH state to play an important role in caries progression [56]. Maybe, *Lactobacillus* could implicate atrophic glossitis development, too.

For fungi, *Saccharomycetales_unidentified_1* was the high relative abundance species at the genus in the atrophic glossitis and significant difference between two groups (Figure 2b, Figure 4b). The result showed that its relative abundance did not rise with the increasing of atrophic degree (Figure 8b). It seems that the increasing with the atrophic degree dose not promote overgrowth of *Saccharomycetales_unidentified_1*. However, the result showed that it was still the main genus in the atrophic

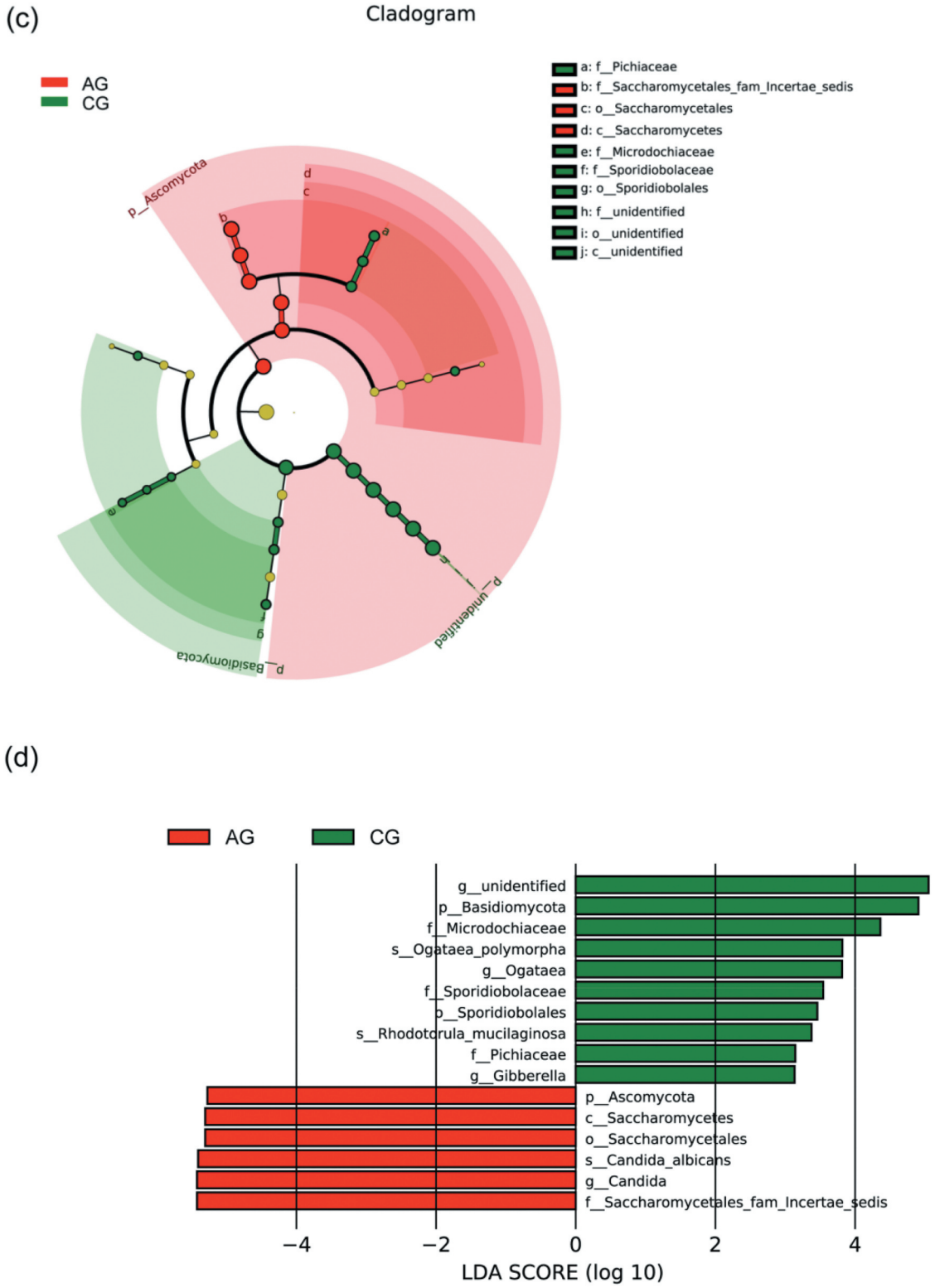

Figure 7.(continued)

glossitis group Figure 7 (c,d). Therefore, *Saccharomycetales* is a vital fungi for atrophic glossitis *and may* influence the progression of atrophic glossitis.

The results showed *Eikenella* have the correlation with the atrophic degree in atrophic glossitis patients (Figure 8a). According to our research, relative abundance of *Eikenella* is very low. This will remind us, whether the low-abundance species would cause changes of the atrophy degree. As we mentioned above, some low-abundance species can be influence the progress of disease.

Above all, the data displayed the community structure and diversity of the oral microbiome in all subjects. Our work showed that *Lactobacillus* and *Saccharomycetales* were the most potent oral microbial genera and were correlated with atrophic

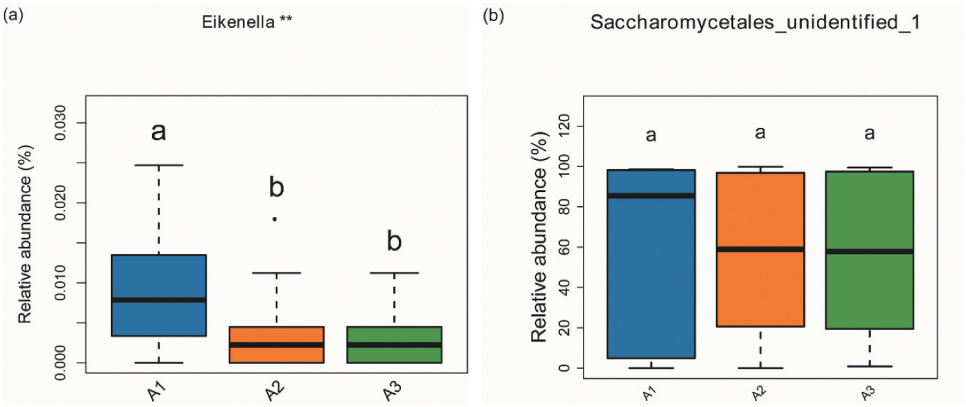

Figure 8. The correlation between the atrophic degree and oral microbiome with a box diagram at the genus level, showing the atrophic degree of some species between different groups and their relative abundance dispersion within the group. A1: slight group (n = 16); A2: moderate group (n = 21); A3:severe group (n = 13). Significantly different groups are indicated with asterisk*. (**a**) Bacteria (**b**) Fungi.

glossitis. However, we do not have enough evidence to indicate that the initiation or progression of atrophic glossitis related to these species. We also cannot completely attribute these pathological phenomena of atrophic glossitis to changes of the specific oral microbiome. On the one hand, few studies of the oral microbiome with atrophic glossitis have been reported. On the other hand, the small sample size may limit our power to detect identified oral microbial species involved with atrophic glossitis. Furthermore, other possible reasons may have influenced the results, including different incidences of atrophic glossitis in different regions and ethnicities, and differences in methodology (i.e. DNA extraction and sequencing technology). In addition, our research makes us speculate that changes occurring in the host may disrupt the harmonious balance with the oral microbiome and that changes in the oral microbiome may also influence disease progression as a risk factor. If we perturb the balance of the oral microbiome, it will result in disease. An unbalanced oral microbiome may be detrimental to general health. Maintaining a healthy oral ecological community is essential to health, and understanding how the host interacts with the oral microbiome has a profound meaning [57, 58]. The relationship between overall health and the balance of the cohabiting groups of common predominant oral microbial genera is a topic that requires further investigation [58]. It is important to note that the relationship with the oral microbiome should be evaluated deeply, and studies of greater scale are necessary before any firm conclusions can be drawn.

Conclusion

This study focused on the salivary microbiome and was based on analysing the composition and diversity of bacteria and fungi in atrophic glossitis patients and healthy individuals, revealing the complex and interconnected relationships of the bacterial-fungal community in the oral cavity. It identified the predominant bacterial and fungal genera related to atrophic glossitis. In addition, more bacterial-fungal species are needed to investigate atrophic glossitis, and the relationship between the oral microbiome and health status should be taken into consideration. This study was expected to provide a clue for bacterial-fungal characterization in atrophic glossitis and attract more attention to the correlations of the oral microbiome and health status.

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Disclosure statement

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