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The niche-specialist and age-related oral microbial ecosystem: crosstalk with host immune cells in homeostasis

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Abstract

Although characterization of the baseline oral microbiota has been discussed, the current literature seems insufficient to draw a definitive conclusion on the interactions between the microbes themselves or with the host. This study focuses on the spatial and temporal characteristics of the oral microbial ecosystem in a mouse model and its crosstalk with host immune cells in homeostasis. The V3V4 regions of the 16S rRNA gene of 20 samples from four niches (tongue, buccal mucosa, keratinized gingiva and hard palate) and 10 samples from two life stages (adult and old) were analysed. Flow cytometry (FCM) was used to investigate the resident immune cells. The niche-specialist and age-related communities, characterized based on the microbiota structure, interspecies communications, microbial functions and interactions with immune cells, were addressed. The phylum *Firmicutes* was the major component in the oral community. The microbial community profiles at the genus level showed that the relative abundances of the genera *Bacteroides*, *Lactobacillus* and *Porphyromonas* were enriched in the gingiva. The abundance of the genera *Streptococcus*, *Faecalibaculum* and *Veillonella* was increased in palatal samples, while the abundance of *Neisseria* and *Bradyrhizobium* was enriched in buccal samples. The genera *Corynebacterium*, *Stenotrophomonas, Streptococcus* and *Fusobacterium* were proportionally enriched in old samples, while *Prevotella* and *Lacobacillus* were enriched in adult samples. Network analysis showed that the genus *Lactobacillus* performed as a central node in the buccal module, while in the gingiva module, the central nodes were *Nesterenkonia* and *Hydrogenophilus*. FCM showed that the proportion of Th1 cells in the tongue samples (38.18% [27.03–49.34%]) (mean [range]) was the highest. The proportion of γδT cells in the buccal mucosa (25.82% [22.1–29.54%]) and gingiva (20.42% [18.31–22.53%]) samples was higher ($P<0.01$) than those in the palate (14.18% [11.69–16.67%]) and tongue (9.38% [5.38–13.37%] samples. The proportion of Th2 (31.3% [16.16–46.44%]), Th17 (27.06% [15.76–38.36%]) and Treg (29.74% [15.71–43.77%]) cells in the old samples was higher than that in the adult samples ($P<0.01$). Further analysis of the interplays between the microbiomes and immune cells indicated that Th1 cells in the adult group, nd Th2, Th17 and Treg cells in the old group were the main immune factors strongly associated with the oral microbiota. For example, Th2, Th17 and Treg cells showed a significantly positive correlation with age-related microorganisms such as *Sphingomonas*, *Streptococcus* and *Acinetobacter*, while Th1 cells showed a negative correlation. Another positive correlation occurred between Th1 cells and several commensal microbiomes such as *Lactobacillus*, *Jeotgalicoccus* and *Sporosarcina*. Th2, Th17 and Treg cells showed the opposite trend. Together, our findings identify the niche-specialist and age-related characteristics of the oral microbial ecosystem and the potential associations between the microbiomes and the mucosal immune cells, providing critical insights into mucosal microbiology.

DATA SUMMARY

All supporting data and protocols have been provided within the article or through supplementary data files. Eight supplementary figures are available with the online version of this article. Raw read sequences of the 16S rRNA gene in this study are publicly available in the NCBI SRA depository, with BioSample accession numbers SUB10380839 and SUB10745111.

INTRODUCTION

The oral microbial ecosystem, with distinct characteristics in structure and function, plays an important role in mucosal homeostasis [1, 2]. The oral mucosa, which consists of various sites [3], e.g. the tongue (specialized), buccal mucosa (lining), gingiva (tooth-associated) and palate (masticatory), is a unique community that has complex and dynamic crosstalk with the commensal microbiome [4, 5].

Fifty years ago, Socransky and Manganiello first reported that the oral microbial communities were distinguishable by site and age [6]. However, this was not fully confirmed for some time due to technological limitations. In 2019, Welch *et al.* proposed the site-specialist hypothesis for the oral microbiota [7] and the original concept of the micrometre-scale structure, which defined the microbial habitats and niches in combination with short- and long-range interactions [8]. To date, characterization of the microbiota in different fields has been performed by high-throughput identification of microbes based on their 16S rRNA genes [9–15]. The importance of spatial organization in microbial ecology has been highlighted in various systems, including the gut microbiota [16], the upper respiratory tract [17] and the oral ecosystem [18–21]. The Human Microbiome Project (HMP), which has assessed distinctive oral habitats, has achieved great progress in recent years [9, 14]. Although these findings present a broad view of the distinctions in human oral habitats, they are currently focused on explaining the role of oral microorganisms in disease [22, 23] rather than that in homeostasis. In addition, characterization of baseline microbial and functional diversity in the oral microbiome has been discussed, yet the taxon–taxon relationships and the role of the microbes themselves in the host mucosa still lack sufficient discussion.

Ageing is a complex, multifaceted process leading to widespread functional decline that affects every organ and tissue [24, 25]. An ageing niche is associated with a wide variety of features at the molecular, cellular and physiological levels [26, 27], which also influences the dialogue with inherent microorganisms. The risk of oral diseases increases dramatically through the course of life [28], to which it is argued the microorganisms contribute [2, 29]. However, it is not clear how ecotypes change with the normal ageing process. While data regarding changes in human oral microbes with ageing are lacking, Yatsunenko *et al.* have characterized the microbial composition of the human gut microbiome across age and geography [30]. Although we do not have direct proof that the same events occur in the oral cavity, this finding would not be surprising [5, 31, 32]. Koren *et al.* interrogated the dynamics of this relationship in the mouth during early life and found that highly tissue-specific responses facilitate maturation [1]. However, the current literature seems insufficient to draw a definitive conclusion about a possible impact of ageing on the oral microbiota [31, 33]. A better understanding of the oral microbiota with ageing is a crucial step for the development of better strategies for the prevention and treatment of oral diseases.

To withstand a wide variety of microbial and mechanical challenges, the immune system of the oral mucosa is composed of tissue-resident and specifically recruited leukocytes [34, 35]. These leukocytes facilitate the establishment and maintenance of local homeostasis but are also capable of causing oral pathologies when unrestrained [36, 37]. Conventional T cells can be classified as major histocompatibility class II (MHC II)-restricted and αβ T cell receptor (TCR)-expressing CD4$^+$ T cells (helper T cells, or Th cells) [38]. Another unique subset of T cells expresses either αβ or γδ TCRs and mostly expresses CD8αα homodimers but not CD8 αβ heterodimers, i.e. unconventional CD8αα$^+$ αβ T cells and γδ T cells [39]. The first encounter between mucosal barriers and the microbiota initiates host–microbiota feedback loops instructing the tailored development of both the immune system and microbiota at each mucosal site. Once established, balanced immunological interactions enable symbiotic relationships with the microbiota in adult life. This process has been extensively investigated in the mammalian epithelial monolayer epithelium-covered intestine and lung mucosae [40–43]; however, the mechanisms utilized by the oral mucosa to establish homeostasis are unclear [1]. In addition, ageing of the immune system (immunosenescence) combined with a low-grade inflammation that develops during ageing (inflammaging) is thought to impact the outcomes of the oral disease challenge from the microbiota [31, 44]. However, the relative importance, mechanistic interrelationships and hierarchical order of these features have not been clarified.

This work aimed to compile an oral microbial community atlas at different oral biogeography and life stages in a mouse model. The study entailed a comprehensive characterization of the microbiomes, their inner interactions and the crosstalk with mucosal immune cells in oral homeostasis in adult and old individuals, which focused on discovering cell types and functions associated with promoting homeostasis and disease susceptibility at the mucosal barrier. We demonstrated that the oral microbial ecosystems were niche-specialist and age-related by assessing not only the community structure but also the taxon–taxon relationships and the predicted functional roles. Furthermore, we characterized correlations between the oral microbiota and tissue-resident immune cells (particularly CD4+ Th cells and γδT cells) probably involved in oral homeostasis.

Impact Statement

To our knowledge, this is the first study to date to characterize oral microbiome communities in four oral niches (tongue, buccal mucosa, keratinized gingiva and hard palate) and at two major life stages (adult and old) in a mouse model and analyse their crosstalk with mucosal immune cells in the context of oral homeostasis. Our findings expand knowledge in the field of oral microbiology that the oral microbiota is niche-specialist and age-related. The microbiota provides a strong microbiological barrier to support mucosal homeostasis. However, health-compatible microbiomes tend to shift to disease-related microbiomes with ageing. First, in adult mice, the oral microbiomes have less of an effect on the distribution of the mucosal immune cells. In old mice, the oral microbial communities are strongly correlated with the immune cells. Second, Th1 cells in the adult group, and Th2, Th17 and Treg cells in the old group are the main immune factors that have a significant influence on the oral microbiota. Together, our findings fill the knowledge gap of the oral microbiota with regard to biogeography and ageing and their crosstalk with mucosal immune cells.

METHODS

For greater details on the study design, sample collection, preparation of samples, DNA extraction, quantitative PCR (qPCR), 16S rRNA gene sequencing and bioinformatics, please refer to the supplementary text (available in the online version of this article).

Oral sample preparation

Wild-type (WT) C57BL/6J male mice were maintained in the barrier facility at the Sun Yat-sen University animal centre, and all experiments were carried out in accordance with institutional guidelines. The age of the mice was strictly selected according to previous publications [45–51], with 3-month-old mice used as the mature adult group and 18-month-old mice used as the old group. Two experiments were carried out: one was performed only using adult mice, which investigated biogeography (including tongue, buccal mucosa, keratinized gingiva and hard palate) [3, 52] and therefore took multiple samples from the same mouse (*n*=5, 20 samples in total); and another experiment investigated the effect of ageing, and therefore only took saliva samples (*n*=5, 10 samples in total), which were not evaluated in the biogeography experiment. The saliva was collected by using Isohelix SK-1 Swabs with Dri-Capsules (Boca Scientific) according to a previous protocol [53]. Samples were snap frozen in liquid nitrogen and then stored at −80 °C until use.

Bacterial DNA extraction, PCR amplification, library preparation and sequencing

Bacterial DNA was extracted and purified using the PowerSoil kit (Mobio) for the mucosal samples, while the Isohelix DNA isolation kit (Boca Scientific) was used for the saliva samples [53]. The V3V4 regions of the 16S rRNA gene were amplified using specific primers (338F and 806R) with a 12 bp barcode. Sequencing libraries were generated using the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England Biolabs) following the manufacturer's recommendations, and index codes were added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Fisher Scientific). Finally, the library was sequenced on an Illumina Nova6000 platform, and 250 bp paired-end reads were generated (Guangdong Magigene Biotechnology).

Processing of the sequencing data

Fastp [54] (version 0.14.1, https://github.com/OpenGene/fastp) was used to control the quality of the raw data by a sliding window (-W 4 M 20). The primers were removed by using Cutadapt software (https://github.com/marcelm/cutadapt/) according to the primer information at the beginning and end of the sequence to obtain the paired-end clean reads. Paired-end clean reads were merged using usearch-fastq_mergepairs [55] (V10, http://www.drive5.com/usearch/) according to the relationship of the overlap between the paired-end reads. When at least a 16 bp overlap was generated from the opposite end of the same DNA fragment, the maximum mismatch allowed in the overlap region was 5 bp, and the spliced sequences were designated as tags.

OTU (operational taxonomic unit) cluster and species annotation

OTUs were clustered by Usearch (UPARSE-OTU algorithm, http://www.drive5.com/usearch/) with default parameters (97% identity) [56]. For each OTU representative sequence, the Silva (https://www.arb-silva.de) database was used to annotate taxonomic information via usearch-sintax (the confidence threshold was set to default, ≥0.8). The taxonomy of the species annotation was divided into seven levels: kingdom, phylum, class, order, family, genus and species. During the clustering, Usearch was able to remove chimaera sequences and singleton OTUs at the same time. The OTUs that were annotated as chloroplasts or mitochondria (16S amplicons) and could not be annotated to the kingdom level were removed.

Removal of contaminants

Contaminant OTUs were identified based on a statistical model using the Decontam package in R [57], using the 'frequency' method to identify contaminants at a threshold of *P*<0.1. The frequency of a contaminant sequence feature is expected to be inversely proportional to the input DNA concentration, as contaminating DNA will make up a larger fraction of the total DNA in samples with very little total DNA. In this method, the distribution of the frequency of each sequence feature as a function of the input DNA concentration is used to identify contaminants. An OTU taxonomy synthesis information table (OTU_table) was then obtained for the final analysis.

Data analysis

Alpha diversity was used to analyse the complexity of species diversity for a sample through three indices (Chao1, Simpson and Equitability indices). The differences between groups were analysed based on the alpha diversity index using R software. Beta diversity analysis was used to evaluate differences between samples in species complexity through Bray–Curtis in the R software. Principal coordinate analysis (PCoA) was performed to obtain principal coordinates and visualize complex and multidimensional data by the vegan package in R. We used analysis of similarity statistics (ANOSIM) in R software to analyse the difference in community structure between groups and whether the differences were significant. LDA effect size (LEfSe) analysis was used to find biomarkers in each group based on the OTU_table. Linear discriminant analysis (LDA) was used to evaluate the impact of significant species (LDA score) by setting the LDA score ≥2. Network prediction analysis between species based on the OTU_table was constructed and analysed by R software and visualized by Cytoscape 3.8.2 (http://cytoscape.org/). The abundance OTU_table was standardized by PICRUS to remove the influence of copies of the 16S marker gene in the genome of species, and then the Greengene ID corresponding to each OTU was compared to the COG database to obtain cog family information. Spearman correlation analysis was conducted using the R package corrplot version 0.84, with an asterisk denoting a significant association (*P*≤0.05). Redundancy analysis (RDA) was used to explore how immune factors influence microbial taxa, while linear regression analysis (LRA) was used to explore how microbial taxa affect immune cells.

Flow cytometry (FCM) analysis

Biopsy preparation into a single cell suspension of oral mucosal tissues was performed as previously described [58]. Data were analysed with FlowJo software (FlowJo v10.8; TreeStar). Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS, version 25.0). The Kolmogorov–Smirnov test and normal Q–Q plots were used for tests of normality. The Levene test was used to determine the homogeneity of variance. One-way ANOVA and Student's *t*-test for parametric statistical tests, and the Kruskal–Wallis rank sum test and Wilcox rank sum test for non-parametric statistical tests were selected based on the data characteristics of normality and homogeneity of variance.

Data availability

Raw read sequences of the 16S rRNA gene in this study are publicly available in the NCBI SRA depository, with BioSample accession numbers SUB10380839 and SUB10745111.

RESULTS

Niche-specialist microbial communities in oral habitats

Niche sharing among oral bacteria and distinct core taxa at different sites

Fourteen microbial contaminants (Fig. S1) were filtered out before conducting the following analyses. A total of 938 OTUs (2.952 million sequences in 20 samples) were defined, including 876 OTUs belonging to 278 genera and 62 OTUs of unclassified genera (Fig. S2a). The microbial richness of the four site samples showed no statistical significance (median Chao1 index >250, Fig. 1a). Palatal and buccal samples were more diverse (median Simpson index <0.4, Fig. 1b) and more even (median Equitability index >0.4, Fig. 1c) than the gingiva and tongue samples. The coordination of the Bray–Curtis PCoA plot (measure of beta-diversity) showed that bacterial communities formed four unique clusters that corresponded to their site (Fig. 1d). The phylum *Firmicutes* was the major component in the four oral microbial communities (Fig. S2b). The order *Bacteroidales* was richer in the gingiva, while *Vellionellales* and *Erysipelotrichales* were more enriched in the palate (Fig. 1e). Further analysis of the microbial community profiles was conducted at the genus level (Figs 1f and S2c). The relative abundances of the genera *Bacteroides*, *Lactobacillus* and *Porphyromonas* were enriched in the gingiva. The abundance of the genera *Streptococcus, Faecalibaculum* and *Veillonella* was increased in the palatal samples, while the abundance of *Neisseria* and *Bradyrhizobium* was enriched in the buccal area. LEfSe analysis showed that 53 OTUs were overabundant in the gingiva, 66 taxa were overabundant in the palate, 215 taxa were overabundant in the buccal tissue and 43 taxa were overabundant in the tongue (Fig. S2d, only the top 20 OTUs in each group are listed). OTUs with a relative abundance >0.1 were selected for investigation as biomarkers. The genus *Streptococcus* was more enriched in the buccal mucosa and palate (Fig. 1g), while *Lactobacillus* had a high abundance in the gingiva and tongue
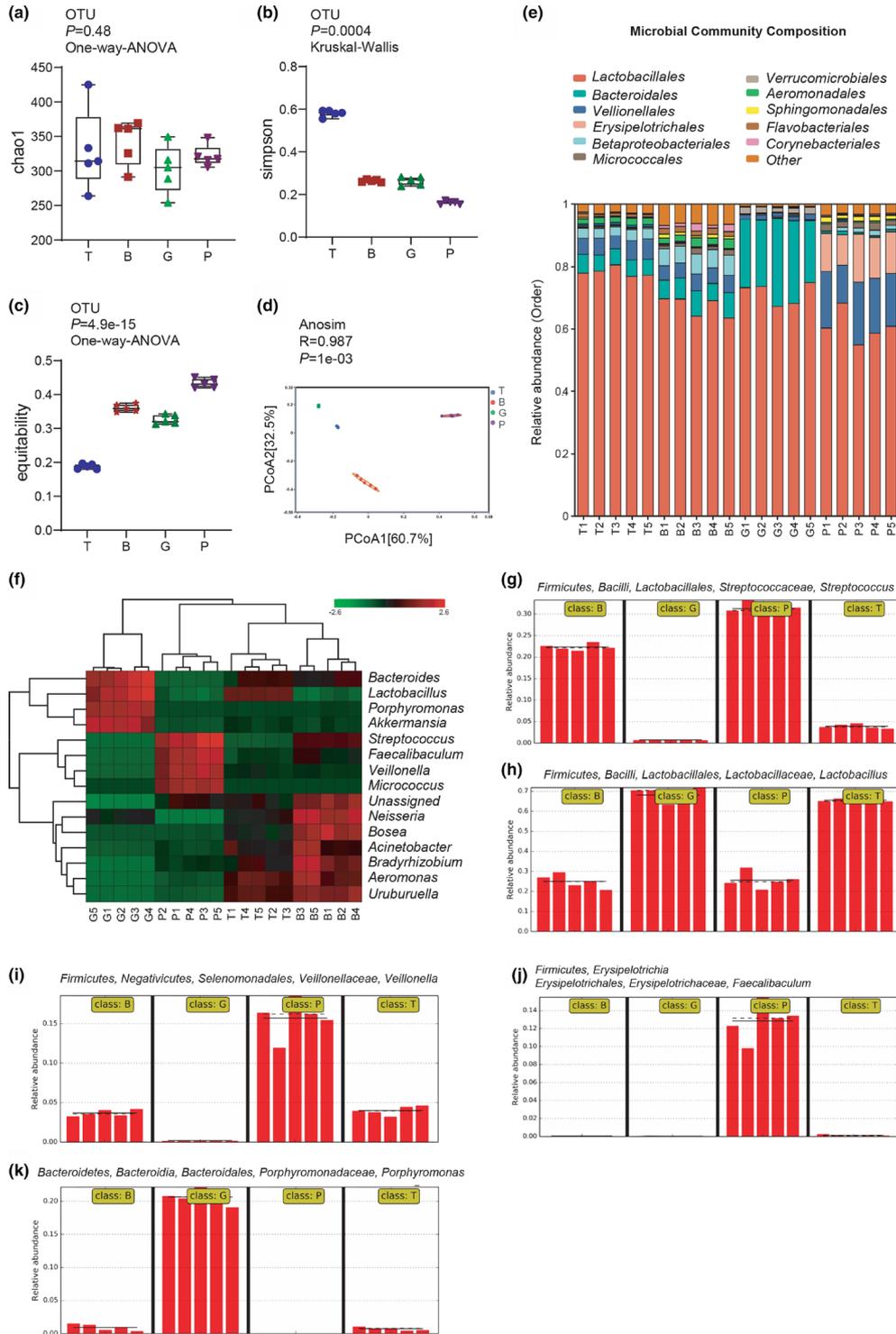
Fig. 1. Niche sharing among oral bacteria and distinct core taxa at different sites. (a–d) Comparative alpha diversity (a–c) and beta diversity (d) principal coordinate analysis (PCoA) of the oral microbiome in different oral sites. (a) Box plot of community richness analysis (Chao1 index). (b) Box plot of community diversity analysis (Simpson index). (c) Box plot of community evenness analysis (Equitability index). Following the normal distribution and homogeneity of variance, significant changes in Chao1 indices and Equitability indices between the different site groups were verified by one-way ANOVA. Since the Simpson indices of each sample did not follow the homogeneity of variance, significant changes were verified by the Kruskal–Wallis rank-sum test. $P \le 0.05$ was used as a threshold. (d) Bray–Curtis PCoA plot (ANOSIM, $R = 0.987$, $P < 0.05$). (e) Bar graph representing the relative community composition of the top 12 orders of the oral microbiota at each oral site. (f) Richness heatmap of the microbiota composition of the top 15 genera within each sample (horizontal axis). Dendrogram of sample sites based on the community similarity along the left axis. The colour of the spots represents the relative abundance of each genus. The taxonomic assignment is shown on the right. Each column represents one subject. (g–k) Differences in relative abundance at the genus level among groups according to LEfSe analysis.

(Fig. 1h). *Veillonella* and *Faecalibaculum* were richer in the palate (Fig. 1i–j), while *Porphyromonas* was specific to the gingiva (Fig. 1k). Together, although different oral sites shared a major part of the microbiota, each oral site had its specific microbiomes.

Interspecies communications and core pathways of the niche-specific oral microbiota

We argue that the microorganisms restricted to each habitat result from the crosstalk of microbes at the unique site, leading to highly specific taxon–taxon interplay. Therefore, network analysis was performed for the top 60 genera to evaluate this suggestion. The results showed a total of 164 negative correlation links in the four modules (Fig. 2a–d) and only 10 positive correlation links in the gingiva module (Fig. 2c). Next, we were interested in which candidates were important to the maintenance of the structure and function of the community. The genus *Lactobacillus* was found to serve as a central node in the buccal module (Fig. 2b), while in the gingiva, the central node comprised the genera *Nesterenkonia* and *Hydrogenophilus* (Fig. 2c). The palate module showed the fewest links among taxa (Fig. 2d). The prevalence of a taxon function across niche-related microbial communities can be explained by either the broad taxonomic distribution of the function or specific enrichment of the function among taxa inhabiting that niche (possibly because the function is selectively advantageous there) [9]. We then identified six site-enriched core pathways from the tongue group, eight from the gingiva area, two from the buccal mucosa and nine from the palate by COG pathways analysis (Fig. 2e). Defence-mechanism-related OTUs were concentrated on the tongue, possibly because of its potential damage during mastication. OTUs that were enriched in the gingiva performed cell wall/membrane/envelope biogenesis, intracellular trafficking secretion, vesicular transport and cell motility functions. Palate samples showed increased OTUs related to extracellular structures. These findings suggest that the unique features of a local environment led to the particular characteristics of its inhabitants.

Correlation analysis between immune cells and the oral microbiomes

Immunosurveillance networks operating at barrier sites are tuned by local tissue cues to ensure effective immunity. Site-specific commensal bacteria provide key signals ensuring host defence in the skin and gut [59–61]. However, how the oral microbiome and tissue-specific signals balance immunity and regulation in the oral mucosa remains minimally explored. FCM showed that the proportion of Th1 cells in the tongue samples (38.18% [27.03–49.34%]) (mean [range]) was higher than those in the gingiva (7.79% [4.92–10.67%], $P<0.01$) and palate (7.19% [3.62–10.76%], $P<0.01$) samples (Figs 3a and S3a). The proportion of $\gamma\delta$T cells in the buccal mucosa (25.82% [22.1–29.54%]) and gingiva (20.42% [18.31–22.53%]) samples was higher ($P<0.01$) than those in the palate (14.18% [11.69–16.67%]) and tongue (9.38% [5.38–13.37%] samples (Fig. 3E and S3e). In addition, the proportions of Th2, Th17 and Treg cells showed no significance at any oral site (Figs 3b–d and S3b–d). A heatmap of Spearman's rank correlation coefficients showed that Th1 cells had a negative correlation with the genera *Neisseria* and *Acinetobacter*. $\gamma\delta$T cells showed a negative correlation with *Neisseria* (Fig. 4a). Furthermore, we aimed to assess the crosstalk between the microbial taxa and immune factors. Thus, RDA was used to explore how immune factors influence microbial taxa, while LRA was used to explore how microbial taxa affect immune cells. The results revealed that Th1 and $\gamma\delta$T cells were the two main immune factors that influenced the oral microbiota in homeostasis (Fig. 4b). However, the whole balanced oral microbiome had less influence on the distribution of the mucosal immune cells (Fig. 4c–g). Overall, these results imply that Th1 and $\gamma\delta$T cells might play an important role in the composition of the oral microbiota.

Age-related microbiome communities in the major life stages

Distinctive core species at each life stage

Nine microbial contaminants (Fig. S4) in saliva samples of the age-related groups were filtered out before conducting the following analyses. A total of 629 OTUs (709000 sequences in 10 samples) were defined, including 442 OTUs belonging to 265 genera and 187 OTUs of unclassified genera (Fig. S5a). Our findings revealed that the community richness was higher in the old mouse samples than that in the adult samples ($P<0.05$, Fig. 5a), while community diversity and evenness showed no significant difference ($P\geq0.05$, Fig. 5b, c). The coordination of the Bray–Curtis PCoA plot (beta diversity measure) formed two clustering groups (Fig. 5d). From the bar graph representing the relative community composition, *Fimicutes* was the most common phylum in the saliva samples (Figs 5e and S5b). A richness heatmap revealed that some opportunistic pathogens, such as *Corynebacterium* [62], *Stenotrophomonas* [63], *Streptococcus* [64–66] and *Fusobacterium* [67, 68], were proportionally enriched in the old samples, while *Prevotella* and *Lacobacillus* were enriched in the adult samples (Figs 5f and S5c). LEfSe analysis showed that 29 OTUs were overabundant in the adult group, while 107 taxa were overabundant in the old group (Fig. S5d; only the top 20 OTUs in each group are listed). Notably, several biomarkers with potential pathogenicity, such as *Enterococcus* [69] and *Fusobacterium* [67, 68], were significantly increased in the old group (Fig. 5g–j). These data indicate that species abundance, rather than diversity, contributes to the observed differences in salivary microbiota between the adult and old groups.

Interspecies crosstalk and functional role of the oral microbiota in the phases

Network analysis showed that both antagonisms and synergies occurred in the oral ecosystem. *Christensenellaceae_R-7.* served as the central node in the young group (Fig. 6a), while fewer links and a lower level of centralization were found in the old group (Fig. 6b). Differences in the predicted COG functional pathways were observed in the saliva microbiota of adult and old mice
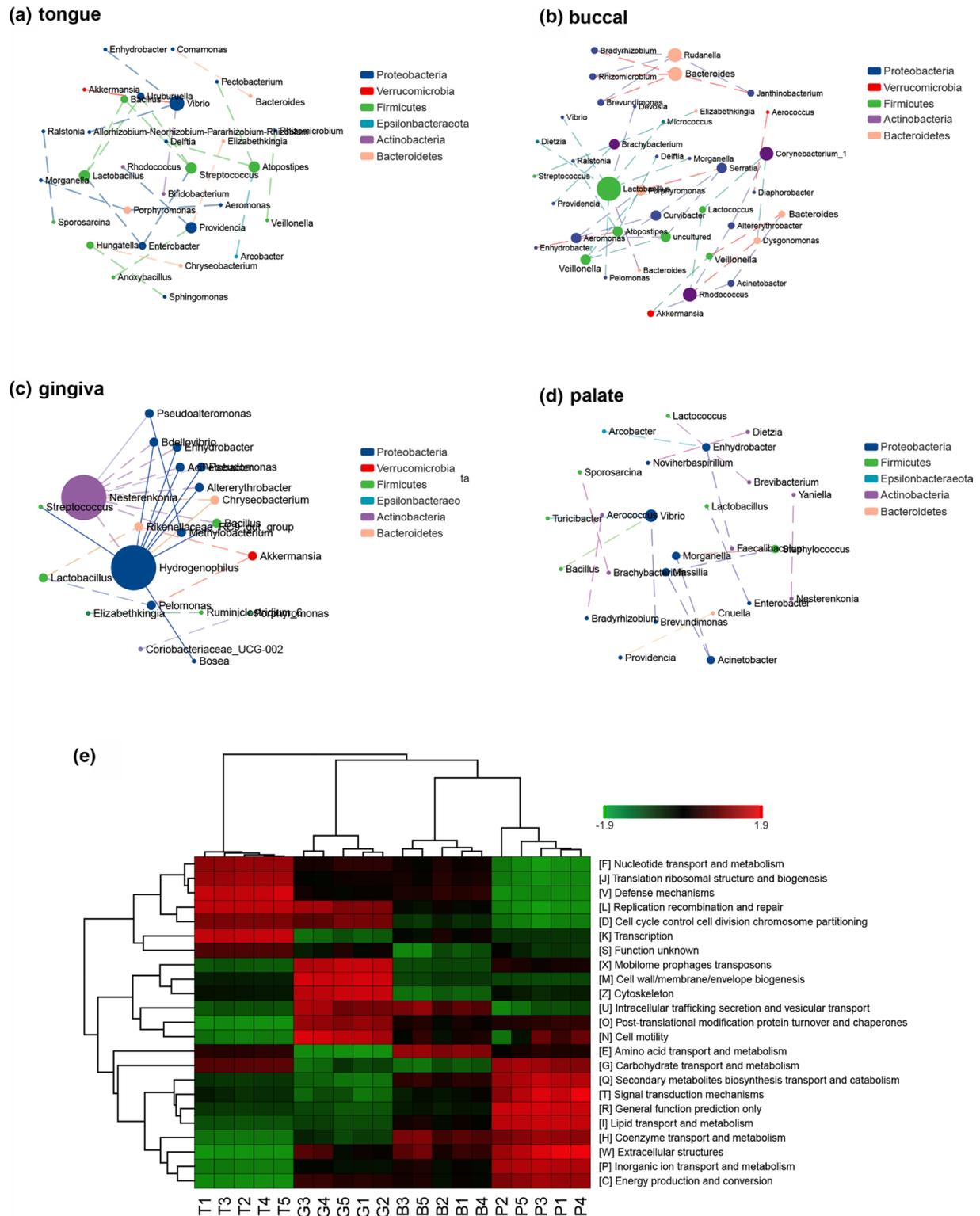
Fig. 2. Genus–genus relationship and functional analysis of different communities. (a–d) Overview of modular structures of sample groups. Network analysis was performed at the level of the top 60 genera. Each node represents a genus, size represents the number of its connections, different colours represent different classifications, the line between the nodes represents the correlation between the two genera, the solid line represents a positive correlation, the dashed line represents a negative correlation, and the thickness of the line represents the absolute value of the correlation coefficient. (a) Network analysis for the tongue samples. (b) Network analysis for the buccal samples. (c) Network analysis for the gingival samples. (d) Network analysis for the palatal samples. (e) COG functional pathway abundance analysis of the oral microbiota in different habitats. G, G1–5: gingiva, T, T1–5: tongue, B, B1–5: buccal mucosa, P, P1–5: palate.
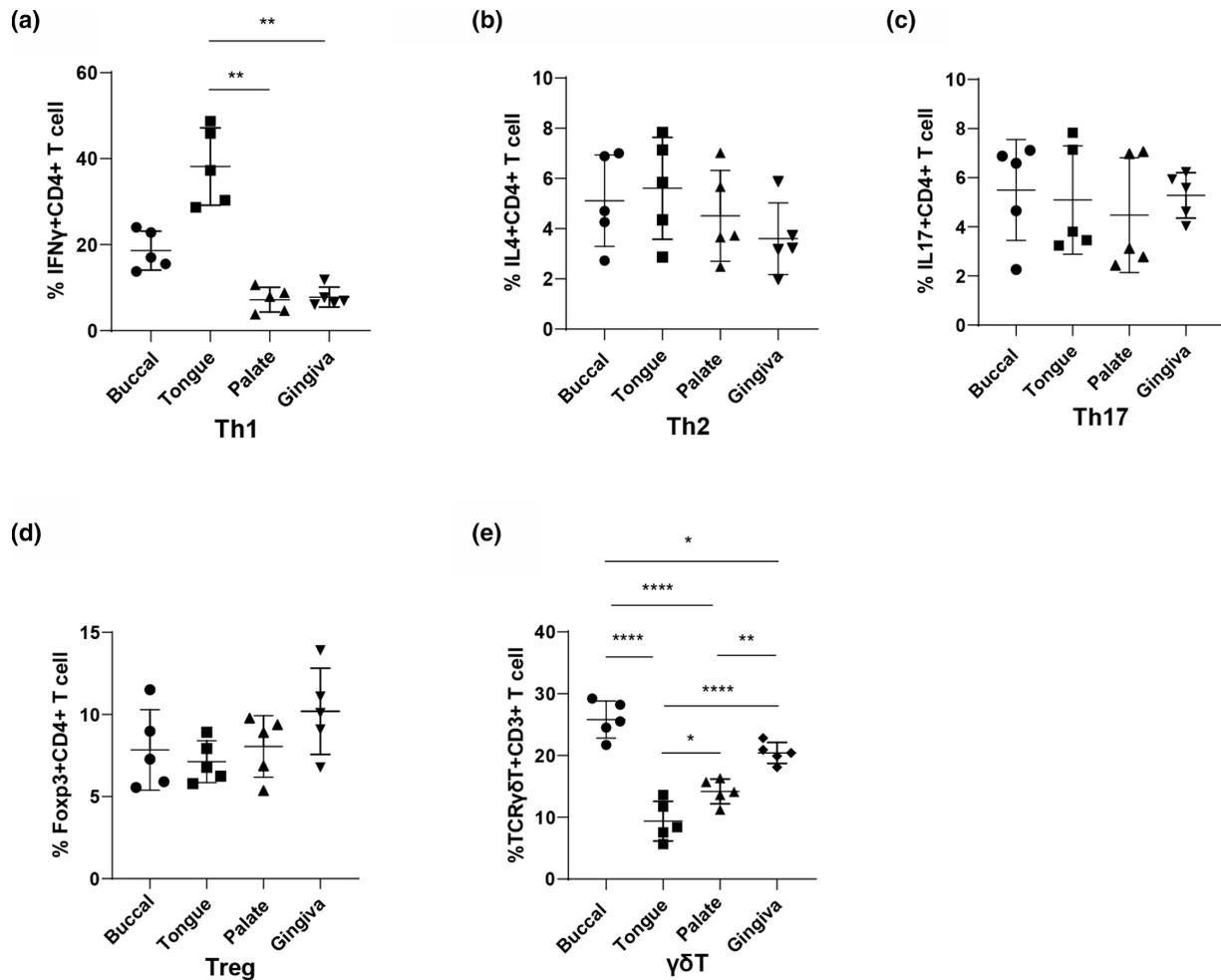
Fig. 3. Boxplots of the abundance of CD4⁺ Th cells and γδT cells distributed at different oral sites. (a) Boxplots for Th1 cells. (b) Boxplots for Th2 cells. (c) Boxplots for Th17 cells. (d) Boxplots for Treg cells. (e) Boxplots for γδT cells. Significant differences in Th2, Treg and γδT cells between groups were analysed with one-way ANOVA. Significant differences in Th1 and Th17 cells between groups were analysed with the Kruskal–Wallis rank sum test. Continuous variables are presented as means±SD. *$P<0.05$, **$P<0.01$, ***$P<0.001$ and ****$P < 0.0001$. More details of the statistical analysis can be found in the supplementary text.

(Fig. 6c). The abundance of several metabolic pathways increased remarkably in adult mice, while cell period pathways, such as cell cycle control, cell division, chromosome partitioning, replication, recombination and repair, were increased in old mice. Together, these results suggest that there are changes in the oral microbiota interplay and microbiome-signalling pathways during ageing.

Crosstalk between immune cells and the age-related oral microbiomes

The findings revealed that the proportion of Th1 cells in the adult groups (28.62% [21.45–35.8%]) was higher than that in the old groups (11.59% [9.82–13.37%], $P<0.01$, Figs 7a and S6a), while the proportion of Th2 (31.3% [16.16–46.44%]), Th17 (27.06% [15.76–38.36%]) and Treg (29.74% [15.71–43.77%]) T cells in the old samples was higher than that in the adult samples ($P<0.01$) (Figs 7b–d, and S6b–d). In addition, the proportion of γδT cells was not significantly different between the two groups (Figs 7e and S6e). Correlations between the relative abundance of bacteria with immune cell subsets, including Th1, Th2, Th17, Treg and γδT cells, are shown in a Spearman correlation heatmap (Fig. 8a). Specifically, Th2, Th17 and Treg cells had a significantly positive correlation with age-related microorganisms such as *Sphingomonas*, *Streptococcus* and *Acinetobacter*, while Th1 cells showed a negative correlation. Another positive correlation occurred between Th1 cells and several inherent microbiomes, such as *Lactobacillus*, *Jeotgalicoccus* and *Sporosarcina*, while Th2, Th17 and Treg cells showed the opposite trend. Remarkably, RDA revealed that Th1 cells in the adult group and Th2, Th17 and Treg cells in the old group were the main immune factors that influenced the composition of the oral microbiota during ageing (Fig. 8b). In addition, at the OTU level, the abundance of old-enriched bacteria correlated positively with the proportion of Th2 (Fig. 8d, $R^2=0.57$), Th17 (Fig. 8e, $R^2=0.61$) and Treg
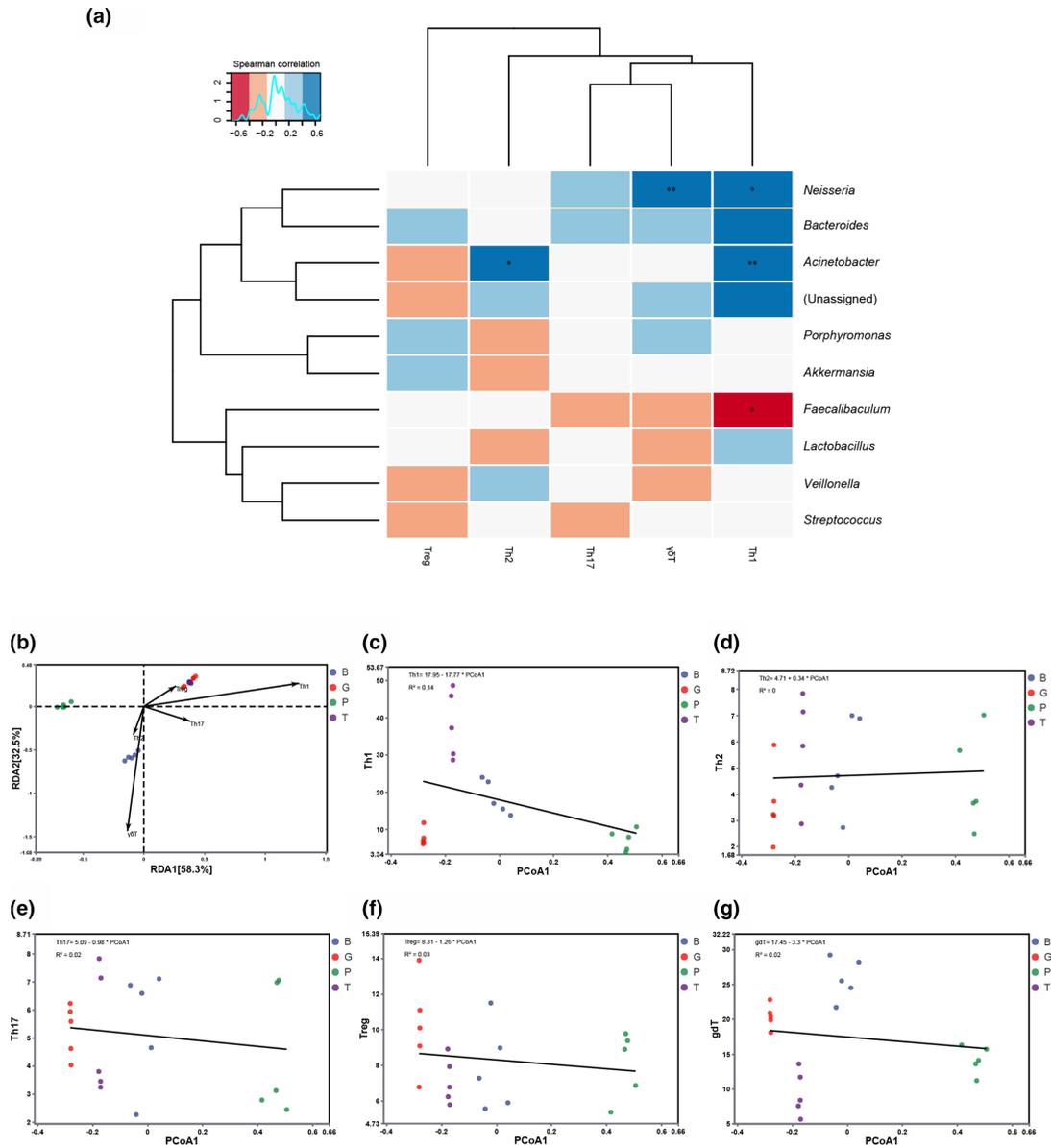
Fig. 4. Correlation analysis between immune cells and the niche-specific oral microbiota. (a) Spearman correlation heatmap showing the correlation between microbial classification (10 dominant genera at the average level) and immune cells. Significant differences between the four groups at: *$P<0.05$ and **$P<0.01$. (b) RDA showing the correlation of immune factors and dominant microbial taxa. Arrows represent different environmental factors. The direction of the coloured arrow indicates the correlation between immune cells and dominant microbial taxa (at the OTU level). Angles between arrows represent their correlation: acute angles indicate that the environmental factors represented are positively correlated, and obtuse angles represent negative correlations. The length of the arrow represents the degree of correlation: the longer the arrow, the greater the influence on the oral microbial communities. (c–g) LRA of the relationship between the immune cells and the oral microbial taxa (at the OTU level), Th1 (c), Th2 (d), Th17 (e), Treg (f) and γδT (g) cells. R^2 indicates the squared correlation coefficient, the *x*-axis represents the oral microbial communities, and the *y*-axis represents immune cells.

(Fig. 8f, $R^2=0.56$) cells and correlated negatively with the proportion of Th1 cells (Fig. 8c, $R^2=0.7$). In short, these findings reveal the tight interplay between the microbiota and immune factors during ageing.

DISCUSSION

The study provides novel insights into the hypothesis of the niche-specialist and age-related oral microbial ecosystem in a mouse model. We identified interspecies communication, microbial function and crosstalk between the oral microbiota and host immune
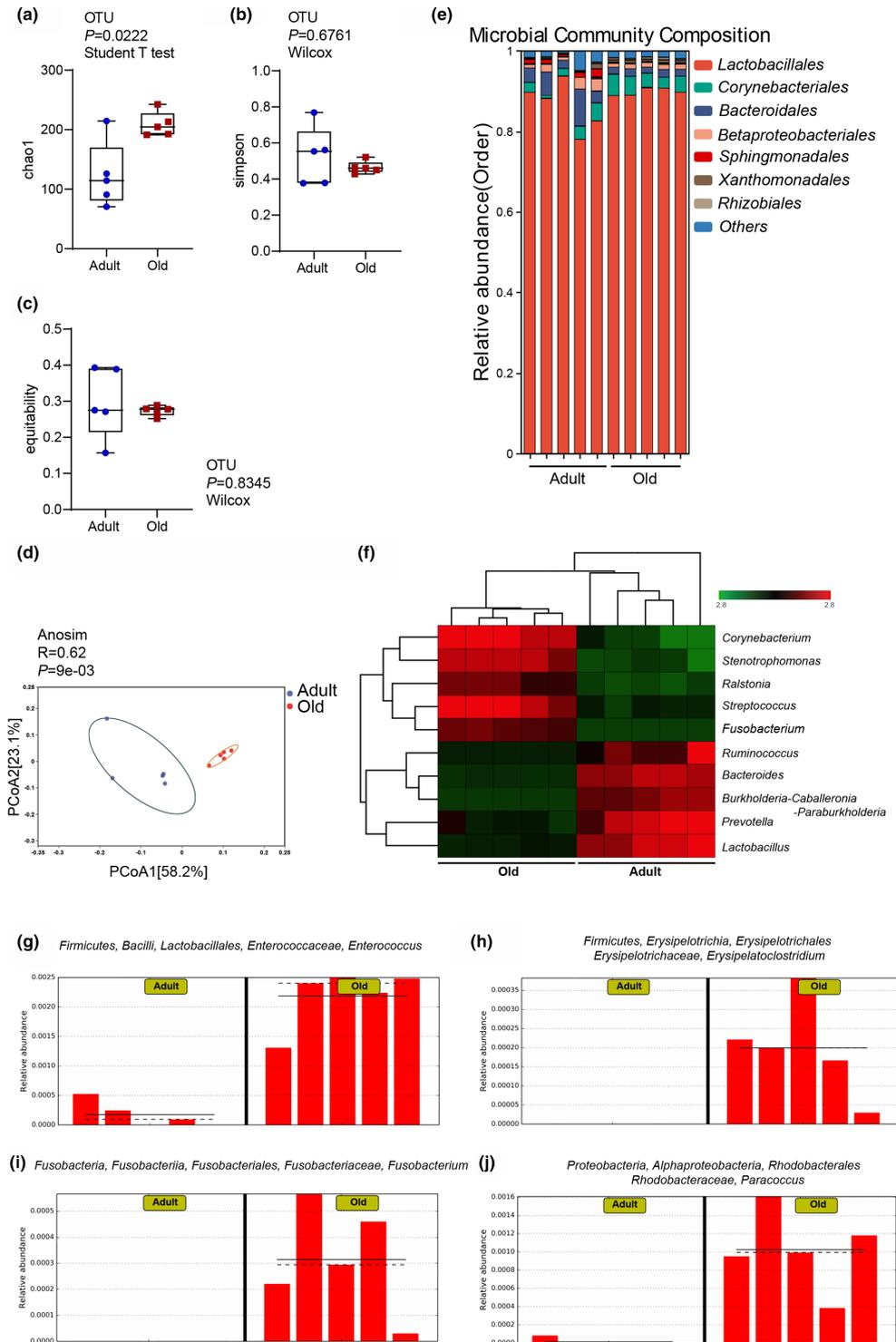
Fig. 5. The oral microbiota community was age-related. (a–d) Comparative alpha diversity (a–c) and beta diversity (d) PCoA of the saliva microbiome between adult mice and old mice. (a) Community richness analysis (Chao1 index). (b) Community diversity analysis (Simpson index). (c) Community evenness analysis (Equitability index). Following the normal distribution and homogeneity of variance, significant changes in Chao1 between the different age groups were verified by Student's *t*-test. Since Simpson indices and Equitability indices of each sample did not follow the homogeneity of variance, significant changes were verified by the Wilcoxon rank-sum test. *P*≤0.05 was used as a threshold. (d) Bray–Curtis PCoA plot (ANOSIM, *R*=0.62, *P*<0.05). (e) Bar graph representing the relative community composition of the top 10 orders in saliva samples of adult mice and old mice. (f) Richness heatmap of the microbiota composition of the top 10 genera within each sample (horizontal axis). Dendrogram of sample sites based on community similarity along the left axis. The colour of the spots represents the relative abundance of the OTUs. The taxonomic assignment is shown on the right. Each column represents one subject. (g–k) Differences in relative abundance at the genus level among groups according to LEfSe analysis.

(a) adult

(b) old

(c)
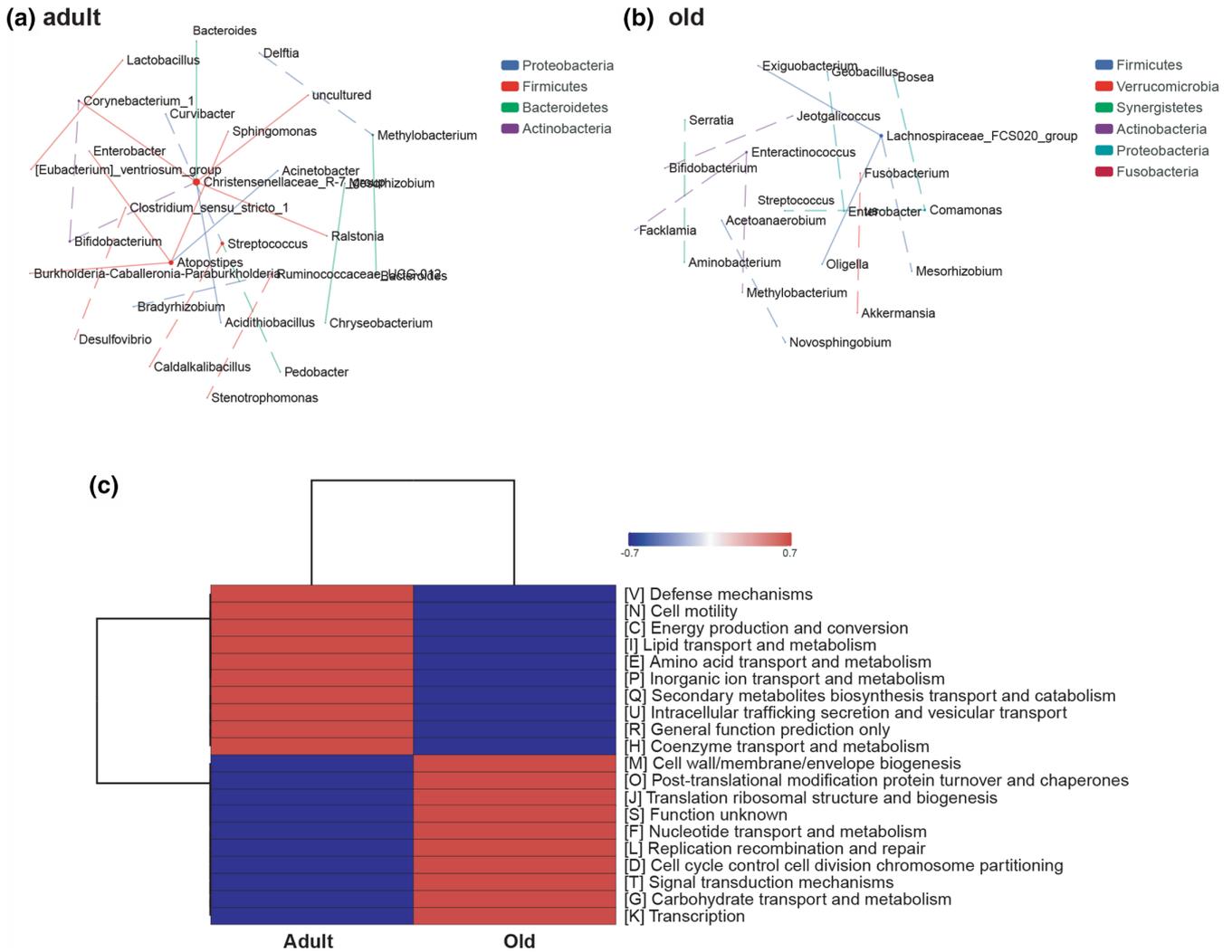
Fig. 6. Taxon–taxon relationship and functional analysis of the oral microbiomes at different stages. (a) Network analysis for the adult samples. (b) Network analysis for the old samples. Network analysis was performed at the level of the top 50 genera. The OTU nodes under each genus were merged into single-genus nodes, which were colour-coded by phyla. The size of the node correlates with the number of links of the node. A solid line indicates a positive correlation. A dotted line shows a negative correlation. (c) COG functional pathway abundance analysis.

cells in different biogeographical regions and at different ages. Our findings demonstrate that the oral microbial ecosystem provides a strong microbiological barrier to support mucosal homeostasis.

Biogeography of the oral microbiome: the niche-specialist hypothesis

The first strength of our work is the meticulous characterization of the oral microbiomes with the strict definition of different oral niches [3]. Four oral mucosal habitats (tongue as the representative of specialized mucosa, buccal mucosa as the representative of lining mucosa, gingiva as the representative of tooth-associated mucosa, and palate as the representative of masticatory mucosa [3]) were selected for study. Our results confirm the hypothesis that the oral microbiota is distinctly enriched at different oral sites [7–9, 14, 57].

Network analysis provides new opportunities for studying biological interactions and habitat preferences. Based on this idea, the taxa that may be most important for maintaining a network can be network hubs, connectors and, perhaps secondarily, modular hubs [70, 71]. The genus *Lactobacillus* in the buccal module (Fig. 2b) and the genera *Nesterenkonia* and *Hydrogenophilus* in the gingiva (Fig. 2c) may be community components that facilitate the stable occurrence of many other taxa. In addition, negative associations also play an important role in community resilience [70]. Network analysis based on genus–genus activities shows that antagonistic interactions are more conserved and enriched in the oral niches. However, it is also important to keep in mind that interpreting negative correlations is problematic because of the compositional bias [71]. Specific enrichment of a function
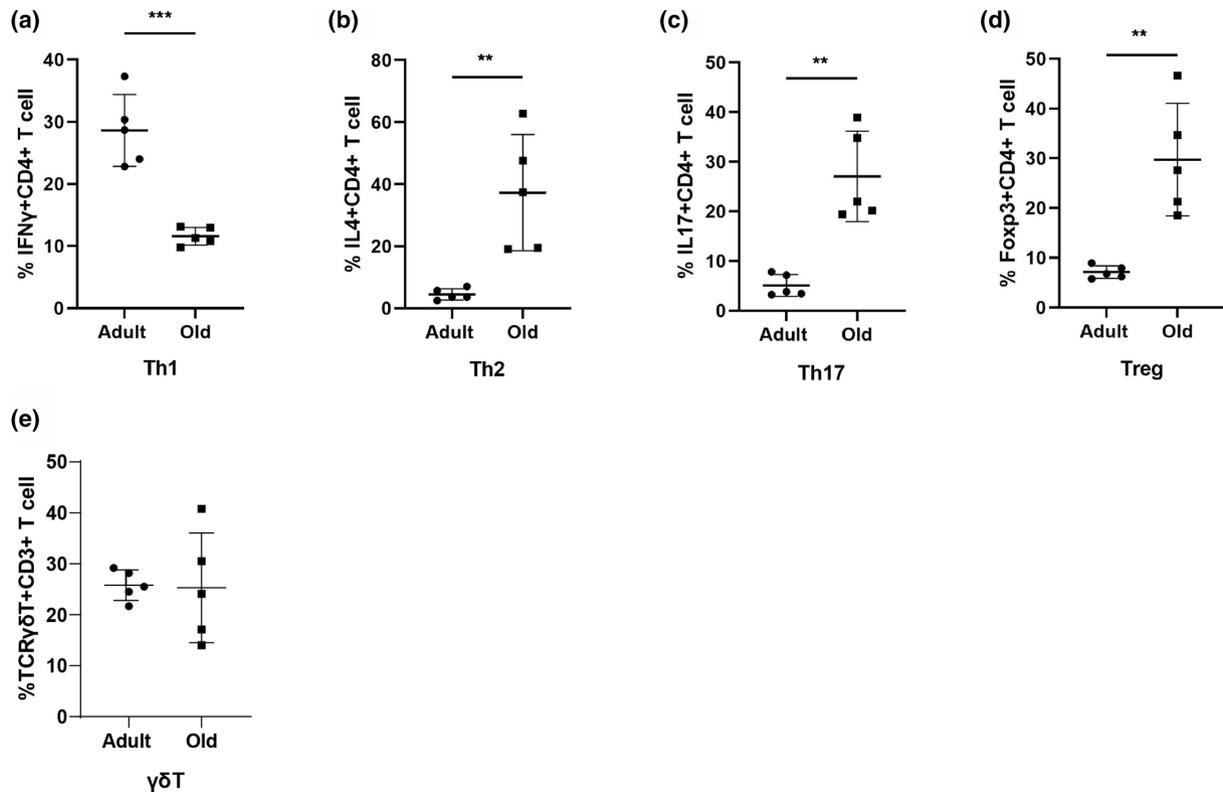
Fig. 7. Boxplots of the abundance of CD4$^+$ T cell subsets distributed at different life stages. (a) Boxplots for Th1 cells. (b) Boxplots for Th2 cells. (c) Boxplots for Th17 cells. (d) Boxplots for Treg cells. (e) Boxplots for γδT cells. Significant differences in Th1 cells between groups were analysed with Student's *t*-test. Significant differences in Th2, Th17, Treg and γδT cells between groups were analysed with the Wilcoxon rank sum test. Continuous variables are presented as means±SD. **$P<0.01$, ***$P<0.001$. More details of the statistical analysis can be found in the supplementary text.

among taxa suggests the functional adaptation by the microbiota to a particular niche within the oral cavity (Fig. 2e). Defence-mechanism-related OTUs were found to be concentrated in the tongue, possibly because of its potential damage during mastication. The marginal gingiva is a 1.5 mm strip of gingival tissue that surrounds the neck of the tooth. This unique microenvironment has abundant nutrients, a proper temperature and humidity and is the best natural habitat for the colonization and proliferation of a great number of oral microorganisms [72]. We found enriched OTUs associated with cell activities and signalling pathways in the gingival niche. In the hard palate, as a masticatory oral mucosa, there were increases in the relative abundance of extracellular structure biosynthetic pathway-related OTUs. This indicates that the most significant factor that determines the niche for a microbe is its local habitat. It also implies that the crosstalk of microbes has led to highly specific niche–microbiome interactions that result in microbes being restricted to a habitat type within the mouth [8, 9, 14].

The microbiota–immune cell axis in different oral niches

The second strength of our study is that the analysis was of recruited immune cells and their crosstalk with the oral microbiota. Recently, a new concept that crosstalk between the oral microbiota and mucosal immunity regulates oral mucosal diseases has been proposed [2]. However, before disease can be fully understood, it is necessary to have a clear understanding of oral homeostasis. Previous studies have shed light on the immunological role of the adult oral epithelium [4]; nevertheless, the spatial and temporal characteristics of immune cells in oral homeostasis remain ill-defined. To our knowledge, this is the first study to date to characterize oral recruited mucosal immune cells in four niches and at two life stages in the context of oral homeostasis.

In the current study, we demonstrated an increase in Th1 cell populations in the adult tongue and buccal mucosa (Fig. 3). Whereas most research on intraepithelial γδT cells has focused on the skin, intestine epithelia and gingiva [39, 73–76], our knowledge of these cells in other oral habitats remains incomplete. Here we not only document a significant recruitment of γδT cells in the gingiva, but also a potential aspect of the high proportion of γδT cells in buccal tissue. Further study will be carried out on the subsets and functions of these γδT cells in buccal tissue. In addition, our results have demonstrated that Th1 and γδT cells were two main immune factors that may influence the stability of the oral microbiomes, while the oral microbiomes had less of an effect on the distribution of the mucosal immune cells in adult mice.
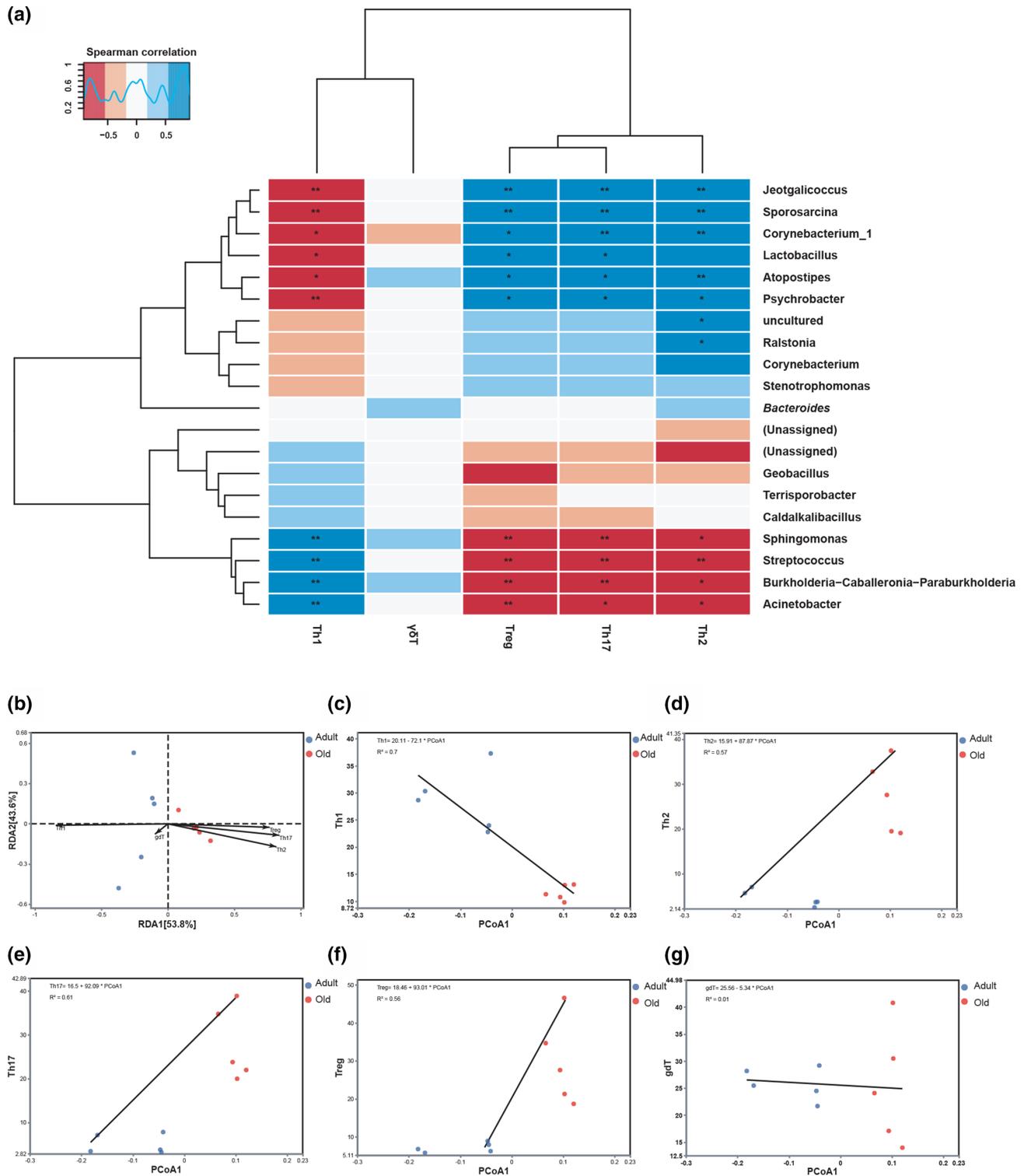
Fig. 8. Correlation analysis between the immune cells and the relative abundance of oral microbiota at different life stages. (a) Spearman correlation heatmap showing the correlation between microbial classification (20 dominant genera at the average level) and immune cells. Asterisks indicate significant differences between the two groups: *$P<0.05$, **$P<0.01$. (b) RDA showing the correlation of immune factors and dominant microbial taxa. Arrows represent different immune factors. The direction of the colour arrow indicates the correlation between immune cells and dominant microbial taxa (at the OTU level). Angles between arrows represent their correlation; acute angles indicate that the environmental factors represented are positively correlated, and obtuse angles indicate negative correlations. The length of the arrow represents the degree of correlation; the longer arrow, the greater the influence on the oral microbial communities. (c–g) LRA of the relationship between the immune cells and the oral microbial taxa (at the OTU level), Th1 (c), Th2 (d), Th17 (e), Treg (f) and γδT (g) cells. R^2 indicates the squared correlation coefficient, the *x*-axis represents the oral microbial communities, and the *y*-axis represents immune cells.

Crosstalk among the oral microbiome: the age-related hypothesis

Time outweighs the effect of host developmental stage on microbial community composition [77]. The third strength is that our findings reinforce the critical role of ageing in oral microbial composition and functions and suggest that there is a trend for disease-associated taxa to increase and health-associated taxa to decrease from birth to old age. Notably, microbial richness was higher in old mice than in adult mice, and the genera *Streptococcus*, *Fusobacterium*, *Corynebacterium*, *Stenotrophomonas* and *Enterococcus* were proportionally enriched in old mice (Fig. 5f–j), which is in accordance with a previous study [78]. Oral pathogenic microorganisms such as *Streptococcus mutans* (as the main bacterial agent for dental caries) [65, 66], and *Fusobacterium nucleatum* (as a periodontitis pathogen) [67, 68] have been detected more frequently in the ageing groups [79]. This evidence highlights that with increasing age, changes in the ecology of the oral cavity are associated with a risk of oral diseases [80].

Each microbe in the mouth is specialized for one life stage or another, such that the microbiota at one period is different from the microbiota at other periods not only in overall composition and proportions of common taxa but also in specific membership [8]. Network analysis was performed to reveal the crosstalk within the microbial communities. The findings supported the idea that the shift of a health-compatible microbiome to a disease-inducing microbiome was due to the proportional increases in pathogenic bacteria with ageing, and not due to *de novo* colonization of disease-associated bacteria in previously healthy individuals [81]. During adult life, the composition and proportions of resident microbes are considered to be reasonably stable, and they coexist in harmony with the host [82]. Physiological changes in ageing include different tissues and functions of the oral cavity [5, 83]. It is conceivable that with ageing, the oral mucosa lacks metabolic activities and increases tissue repair, which is also reflected in the distinguishable enrichment of oral microorganisms. This evidence indicates that the host environment drives changes in the oral microbiome with temporal variation [84]. The context of the individual's environmental exposure offers an opportunity to predict risk for early disease biomarkers.

Crosstalk between the oral microbiota and immune cells with ageing

The ageing process is associated with a low-grade systemic inflammatory status, even in the absence of clinical signs of infections [31]. This phenomenon, known as inflammaging, might interfere with the resident microbial population in the naturally infected areas of the human body, such as the gut and the oral cavity [85, 86]. In addition, the decline in function of the immune system brought on by natural age advancement, generally referred to as immunosenescence [86, 87], has been argued to contribute to the increased susceptibility of elderly individuals to microbial infections, which may further magnify the effects of inflammaging [88]. In the current study, we demonstrated increased populations of Th2, Th17 and Treg cells in the mucosa of old mice (Fig. 7). The fourth strength is that our results detail an atlas of Th cell populations, especially later in life, providing insights into cell functionality in homeostasis and disease susceptibility, and opening avenues for further mechanistic exploration of these subsets [2]. Th cell populations are characterized by the production of different effector cytokines and the expression of distinct transcription factors to defend against pathogens and maintain balance. Th2 cell differentiation is initiated by TCR signalling together with IL-4 and subsequently by STAT6 signal transduction, leading to the expression of the transcription factor GATA-3 [89]. Th17 cells are an important subset of effector T cells that are protective during extracellular bacterial and fungal invasion [90–94]. Treg cells are essential for maintaining self-tolerance and immunosuppression [95]. As discussed above, probably due to the reduction in oral clearance and local antibodies or antimicrobial peptides, several opportunistic pathogens are proportionally enriched in old mice. The increased populations of Th2, Th17 and Treg cells in the mucosa of old mice could be suggested to maintain host immune homeostasis and minimize tissue damage.

The first encounter of mucosal barriers with the microbiota initiates host–microbiota feedback loops that instruct the tailored development of both the immune system and microbiota at each mucosal site. Once established, balanced immunological interactions enable symbiotic relationships with the microbiota in adult life [1]. Hence, we utilized our atlas to assess the crosstalk between the abundance of the oral microbiomes and the recruited immune cells, providing further understanding of the microbiota–immune axis underlying oral mucosal homeostasis during ageing. Th1 cells in the adult group and Th2, Th17 and Treg cells in the old group were the main immune factors that influenced the composition of the oral microbiota during ageing (Fig. 8b). In addition, we showed that Th1 cells had a negative correlation with strictly facultative anaerobic bacteria, and bacteria that may cause opportunistic infections. These findings suggest a previously unacknowledged role for the age-related microbiota in immune responsiveness in oral homeostasis, which is in accordance with recent findings that identify a microbiota–immune axis in ageing of other tissue compartments [4, 96–100]. For example, mucosal colonization of *Porphyromonas gingivalis*, an important component of the dysbiotic oral microbiome [68], can regulate the plasticity of interstitial Th17 and Treg cells, leading to an unfavourable balance that promotes disease [101].

Although our study has several strengths, two limitations are addressed here. The first limitation was that while we identified the association between the oral microbiota and several T cell subsets, such as Th cells and γδT cells, there are still other immune cells that deserve study, such as a unique population of CD3$^+$ CD4/CD8 double-negative T cells known as mucosa-associated invariant T (MAIT) cells, neutrophils and innate lymphoid cells (ILCs) [1]. The second limitation was that to identify the correlation between the oral microbiota and the resident immune cells in different niches and at different

ages, we performed our studies with animal experiments. Research in animal models has been used as a tool for gaining insight into the bacterial contribution to dental caries [64, 65], periodontitis [67, 68] and other oral diseases [102, 103]. The predominant animal models for studying the microbiome are rodents, such as mice. Mice are often preferred because they are well characterized and have many physiological similarities to humans [104]. Several potential oral pathogens of mice are the same as for humans. For example, in mouse models, *Streptococcus mutans* has been used to study the formation of dental caries [64, 65], and *Porphyromonas gingivalis* and *Fusobacterium nucleatum* have been used for studying periodontal disease [67, 68]. However, only male mice were used in our experiments, and caution should be taken when generalizing the results to both sexes [105, 106]. Obviously, animal models will never fully replicate all factors of human biology. There is still a need to conduct clinical studies in humans to gain a holistic understanding of the key features of the oral ecosystem that contribute to oral health.

Author contributions
Conceptualization: D.L. and Z.W. Methodology: D.L., Q.H., L.Y. and W.D. Formal analysis: D.L., Y.X. and J.F. Investigation: D.L., Q.H., L.Y., D.W. and H.L. Resources: all authors. Writing–Original Draft Preparation: D.L. and Q.H. Writing–Review and Editing: all authors. Visualization: D.L. and Z.W. Supervision: Z.W. Project Administration: D.L. and Z.W.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
All experiments were carried out in accordance with institutional guidelines.

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