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LONG PAPER

Oral microbiome variations related to ageing: possible implications beyond oral health

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Abstract
The global population is getting older due to a combination of longer life expectancy and declining birth rates. Growing evidence suggests that the oral microbiota composition and distribution may have a profound effect on how well we age. The purpose of this study was to investigate age-related oral microbiome variations of supragingival plaque and buccal mucosa samples in the general population in Latvia. Our results indicated significant difference between supragingival plaque bacterial profiles of three age groups (20–40; 40–60; 60 + years). Within supragingival plaque samples, age group 20–40 showed the highest bacterial diversity with a decline during the 40–60 age period and uprise again after the age of 60. Among other differences, the important oral commensal *Neisseria* had declined after the age of 40. Additionally, prevalence of two well-documented opportunistic pathogens *Streptococcus anginosus* and *Gemella sanguinis* gradually rose with age within our samples. Furthermore, supragingival plaque and buccal mucosa samples significantly differed in overall bacterial composition.

Keywords Oral microbiome · Microbiota · Human microbiome · Life span · Metagenomics · Ageing

Introduction

The global population is rapidly ageing as a result of declining fertility rates and continued growth of average life span. It is expected that the proportion of population aged 65 years and over might increase from 18% in the year 2000 to 38% by 2050 (Rudnicka et al. 2020). Addressing this demographic transition in a way that promotes healthy life span is important, as it affects almost all aspects of society.

In the elderly, incidence of oral diseases, such as dental caries, soft tissue diseases and periodontal diseases, is increased (Guiglia et al. 2010; Gil-Montoya et al. 2015;

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López et al. 2017). Equally, older age brings forth a wide range of health disorders related to immune and endocrine systems, nervous system, locomotor system and cardiovascular system (Wick et al. 2000). Their pathological foundation, however, is built earlier in life. Growing evidence suggests that the oral microbiome composition, species identity and combinations, density and distribution of bacteria may have a profound effect on how well we age (Shoemark and Allen 2015).

Over the last decade, rapidly advancing human microbiome studies have linked human-inhabiting microbial communities, their members and shifts in their composition to a wide range of health conditions. However, the topic of human oral microbiome, despite its unique accessibility in comparison to other body sites, still remains relatively understudied (Willis and Gabaldón 2020). It is known that oral cavity constitutes a complex, biogeographically diverse ecological environment that changes over time (Mark Welch et al. 2019). Several studies have detected age-related changes in oral microbiota, although findings vary, underlying the complexity of the oral microbiome phenomenon. For instance, Liu et al. investigated microbial composition of three oral sites (gingival crevicular fluid, tongue back and saliva), concluding that bacterial alpha diversity decreases with age, while beta diversity has a tendency to increase (Liu et al. 2020). Willis et al., on the other hand, comparing oral rinse samples across ages, concluded that middle ages represent the most homogenous oral microbiota composition, while older ages are characterized by increased microbial diversity on account of typically low abundance taxa (Willis et al. 2022). Furthermore, a growing number of studies focus on oral microbiome shifts associated with systemic diseases, including age-related conditions: cardiovascular disease (Teles and Wang 2011; Chhibber-Goel et al. 2016), different types of cancer (Fan et al. 2018; Rao et al. 2020; Teles et al. 2020), cystic fibrosis (Willis et al. 2021), celiac disease (Tian et al. 2017; Valitutti et al. 2019), schizophrenia (Qing et al. 2021), neurodegenerative diseases (Shoemark and Allen 2015; Mihaila et al. 2019; Jo et al. 2022) and other disorders. Yet, the variations of oral microbiota with regard to ageing have not been extensively studied.

Exploring age-related characteristics of microbial communities within different sites of oral cavity may present new opportunities for microbiome-based diagnostics, open up new avenues for preventive healthcare and offer innovative treatment options. In this study, we investigated the bacterial profiles of supragingival plaque and buccal mucosa samples of the general population of Latvia across three age groups: 20–40, 40–60 and 60 + years. Our results reveal niche-dependent oral microbiome variations related to different age groups and provide novel insights into possible links between oral microbiome shifts and age-related diseases.

Materials and methods

Supragingival plaque and buccal mucosa sample collection

Supragingival dental plaque samples ($n = 40$, "S" samples) and buccal mucosa samples ($n = 20$, "V" samples) were collected from randomly selected individuals 2 h after a meal by gently rubbing sterile polyester swab over the oral mucosa and tooth surfaces. A questionnaire was handed out to participants to collect data about individual's age, sex and smoking status (Table S1, Supplementary Materials). A pair of samples from both oral locations was collected for individuals Nr. 1–20 (1S, 1 V–20S, 20 V), whereas only supragingival plaque samples were collected for individuals Nr. 21–40. Samples were collected with patient consent. An ethical review and approval of this study was provided by the Research Ethics Committee of Riga Stradins University, decision No 6-3/4/5 (25 April 2019).

DNA isolation and quantification

Total DNA was extracted using the phenol–chloroform method. The procedure involved six steps. The first step was incubation with lysis buffer, which consisted of 500 µl cell suspension solution (CSS), 100 µl 10% sodium dodecyl sulphate (SDS) and 2.5µ proteinase K, which was added to 500 µl sample swab suspension in nuclease-free water. The resulting solution was mixed by vortexing, incubated at 55ºC for 2 h and centrifuged at room temperature at 4 000 rpm for 10 min. The second step involved the transfer of the upper fraction of the solution to a new microtube and addition of 1 ml of phenol. Then, 30 s vortexing and centrifugation with previous parameters followed. Phenol wash was repeated for a second time. The third step involved addition of 1 ml of chloroform to the upper fraction of extraction solution, which was transferred to a new microtube. Vortexing and centrifugation were done as previously described. For the fourth step, the upper layer of extraction solution was once again transferred to a new microtube and 1 ml of isopropanol was carefully added. The solution in the microtubes was carefully mixed by rotating tubes three times. Afterwards, microtubes were placed at − 20 ºC overnight. The fifth step of DNA extraction, which involved centrifugation of microtubes at 4ºC and 12 000 rpm for 15 min, was done the next morning. All supernatant was carefully discarded and the DNA precipitate, which has formed at the bottom of microtube, was once again centrifuged with 1 ml of ice-cold 70º ethanol. After centrifugation, ethanol was

discarded and tubes were kept open in a fume hood for 30 min to evaporate the remaining ethanol. Within step 6, the DNA precipitate was resuspended in 100 µl of low-TE buffer. Quantification of the resultant DNA was done using Qubit dsDNA HS Assay Kit (Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. To evaluate sample contamination from laboratory sources, a control sample (DNA extraction blank) was processed in parallel with oral microbiome samples.

Library preparation and shotgun metagenomic sequencing

Prior to library preparation, all oral microbiome samples underwent the DNA fragmentation step using the Ion Shear™ Plus Reagent Kit (Ion Torrent™, Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's instructions. Fragmentation conditions were selected to achieve the desired fragment size (150–250 bp). Further, size-selection procedures were performed on DNA samples using NucleoMag® NGS Clean-up and Size Select magnetic beads (Macherey–Nagel) to remove fragments larger than 250 base pairs. Sequencing libraries were prepared using Ion Plus Fragment Library Kit (Ion Torrent™) in accordance with the manufacturer's instructions. Prior to sequencing, libraries underwent amplification and quality assessment using an Agilent High Sensitivity DNA Kit and Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). The resultant library concentration was assessed using Qubit dsDNA HS Assay Kit (Life Technologies, Carlsbad, CA, USA) (Table S2, Supplementary Materials). Sequencing was performed on an Ion Proton™ System (Ion Torrent™, Thermo Fisher Scientific, Waltham, MA, USA). The metagenomes data are available in the Sequence Read Archive hosted at the NCBI under the BioProject accession code PRJNA931979.

Sequencing data analysis

Raw sequencing data were initially automatically pre-processed on the local Ion Torrent Proton server with the use of Proton software. Within this pre-processing step filtering of barcodes, sequencing adapters, polyclonal and low-quality sequences took place. Resultant data were exported in the form of BAM files. Further, exported BAM files were quality processed using the Galaxy public server (Kosakovsky Pond et al. 2009; Goecks et al. 2010). Within this procedure, BAM files were converted to fastq. FastQC plugin was used to access and visualize quality data of each individual sequence. Further, FastQ Groomer tool was used to enforce the quality score. Filter by quality tool was applied with quality cutoff value: 20. Further, FastQC tool was operated again to access the quality improvements. Overrepresented sequences, identified as adapter leftovers, were detected and subsequently removed using Trim Galore! tool. Sequencing data were exported in the form of fasta files for further manipulations.

Kraken2 v2.0.7 with the standard Kraken2 database was used for sequencing data taxonomic assignment (Wood and Salzberg 2014; Wood et al. 2019). To compute the abundance of species from a metagenomics sample Bracken (Bayesian reestimation of abundance with Kraken) was utilized (Lu et al. 2017).

Kraken/Bracken taxonomy reports were further manipulated with the use of Pavian R application (Breitwieser and Salzberg 2020), generating quality assignment and preparing data for the next step of statistical analysis and representation, which was done through MicrobiomeAnalyst (https://www.microbiomeanalyst. ca/ (accessed on 16 October 2022)) web application (Dhariwal et al. 2017; Chong et al. 2020). Identification and removal of laboratory contaminants from metagenomics data was performed using an open-source R package decontam (https://github.com/benjjneb/decontam (accessed on 16 October 2022)) (Davis et al. 2018). A hard cutoff of 0.001% abundance was applied to remove species with low abundances.

Statistical analysis

Samples were differentiated by individual's age into three age groups: 20–40 years (*group 20–40*), 40–60 years (*group 40–60*) and 60 + years (*group 60* +). Further, samples were also compared based on individual's smoking status (smokers/non-smokers), individual's sex (male/female) and sampled oral cavity location (supragingival plaque/ buccal mucosa).

Alpha-diversity analysis (*T* test/ANOVA with Shannon diversity index) was used to assess intergroup differences. Beta diversity between sample groups was tested using permutational multivariate analysis of variance (PERMANOVA) and was represented by principal coordinates analysis plots (PCoA). Univariable *T* test (ANOVA) analysis with *p* value cutoff 0.05 was implemented.

The Kruskal–Wallis test and the linear discriminant analysis (LDA) effect size (LEfSe) method were used to analyse intergroup differences (Segata et al. 2011). For this analysis, the false discovery rate (FDR)-adjusted *p* value cutoff was set to 0.05 and the threshold on the logarithmic LDA score for discriminative features was set to 3.5.

Results

Sequencing data

In total, a set of 60 metagenomic DNA samples were analysed: 40 supragingival plaque samples and 20 buccal

mucosa samples (Table S1, Supplementary Materials). A total of 0.12 billion sequences were generated, with an average of 2.04 million reads per sample (standard deviation (SD) = 1.19 million reads). (Table S2, Supplementary Materials) Among them, 9.81% were classified as bacterial species-level reads with an average of 0.20 million reads per sample (6496-764921; SD = 0.19 million reads).

The blank sample showed much lower level of classified reads despite sufficient sequencing depth—only 1356 reads (2.84%) were classified to the bacterial species level. The majority of the reads found in blank sample appeared to be unclassifiable sequencing artefacts. Within the blank sample, 10 bacterial species were detected, with the majority (70.27%) belonging to *Delftia* genus, which were subsequently removed prior to data analysis. (Fig. S1, Supplementary Materials).

Taxonomic analysis of bacterial communities in supragingival plaque samples at the genus level

Bacterial communities in supragingival plaque samples of all three age groups (20–40; 40–60; 60 +) showed that the most abundant bacterial genera corresponded to typical oral taxons (Fig. 1a): *Streptococcus, Haemophilus, Veillonella, Prevotella, Actinomyces, Neisseria, Rothia, Fusobacterium, Leptotrichia* and *Gemella.* The exact proportions, however, varied between sample groups. Alpha-diversity analysis (*T* test/ANOVA with Shannon diversity index) showed statistically significant difference between groups. Age group 20–40 years expressed a significantly higher microbial diversity than the 40–60 and 60+ groups (F value = 4.5036, $p < 0.05$) (Fig. 1b). However, the 60+ age group had a slightly higher bacterial genus diversity than the 40–60 age group.

Beta-diversity analysis of microbial genera composition of the 20–40, 40–60 and 60+ samples in the form of PCoA plots highlighted a significant difference between the groups. ###While all three sample groups did overlap, age groups 40–60 and 60+ formed distinct clusters, indicating that the dissimilarities between microbial profiles within the groups might have increased, while the overall bacterial genera diversity in comparison with young age (20–40) declined, as seen previously in Fig. 1b (PERMANOVA, F value = 1.98, $p < 0.05$) (Fig. 1c).

To identify specific bacterial genera of the oral microbiota which are associated with different age, we used the linear discriminant analysis effect size (LEfSe) method comparing microbial composition of the three age groups (p value cutoff: 0.05, LDA score: 3.5). The identified predominant bacterial genera are shown in Fig. 2, illustrating the largest differences between taxa represented by sample groups. Eight bacterial genera were found to be significantly more abundant in the group 20–40 tears: *Neisseria, Leptotrichia, Lachnoanaerobaculum, Bacteroides, Bacillus, Chryseobacterium, Clostridium,* and *Pasteurella*. There were five bacterial genera associated with group 60+ years: *Propionibacterium, Lactobacillus, Bifidobacterium, Parascardovia* and *Mogibacterium*. Only one bacterial genus was found to be associated with the 40–60 group: *Alloprevotella*.

Taxonomic analysis of bacterial communities in supragingival plaque samples at the species level

Species-level relative abundance of sample groups 20–40, 40–60 and 60+ years demonstrated typical oral bacteria with *Streptococcus mitis* being the most abundant bacterial taxon in all groups, followed by *Haemophilus parainfluenzae*, *Veillonella parvula, Streptococcus pneumoniae, Streptococcus oralis, Prevotella melaninogenica, Haemophilus haemolyticus, Streptococcus gwangjuense, Haemophilus influenzae, Fusobacterium nucleatum* and other species. (Fig. 3a).

In contrast to genus level, there was no significant difference of species alpha diversity between sample age groups 20–40 and 60+ . Group 40–60, on the other hand, demonstrated a significantly lower bacterial diversity than other two groups (*T* test/ ANOVA with Shannon diversity index, $p < 0.05$) (Fig. 3b), whereas beta diversity in the form of PCoA plot (PERMANOVA, F value = 2.5349, $p < 0.05$) on species level resembled genus-level beta-diversity pattern between age groups, displaying all three groups as overlapping, but distinct clusters. (Fig. 3c).

Using linear discriminant analysis effect size (LEfSe) method ((FDR)-adjusted p value cutoff set to 0.05 and LDA score set to 3.5), 17 bacterial species were identified to be significantly different between the age groups. (Fig. 4) Group 20–40 was characterized by five significantly prevalent bacterial species: *Streptococcus sp. ChDC B345, Leptotrichia sp. oral taxon 212, Fusobacterium periodonticum, Capnocytophaga leadbetteri, Leptotrichia hongkongensis, Capnocytophaga sp. ChDC OS43* and *Neisseria sp. KEM232*. Two bacterial species were associated with group 20–40: *Fusobacterium pseudoperiodonticum* and *Alloprevotella sp. E39*. Eight bacterial species were found to be distinctive features of group 60+ : *Streptococcus gwangjuense, Veillonella atypica, Prevotella denticola, Streptococcus sp. 116 D4, Streptococcus anginosus, Treponema sp. OMZ 804, Fusobacterium pseudoperiodonticum, Gemella sanguinis* and *Parascardovia denticolens*.

Taxonomic analysis of bacterial communities in buccal mucosa samples at the genus level

Buccal mucosa sample taxonomic composition also represented typical oral bacterial genera, with a minor difference compared to supragingival plaque sample bacterial composition (Fig. 5a): *Streptococcus, Haemophilus,*

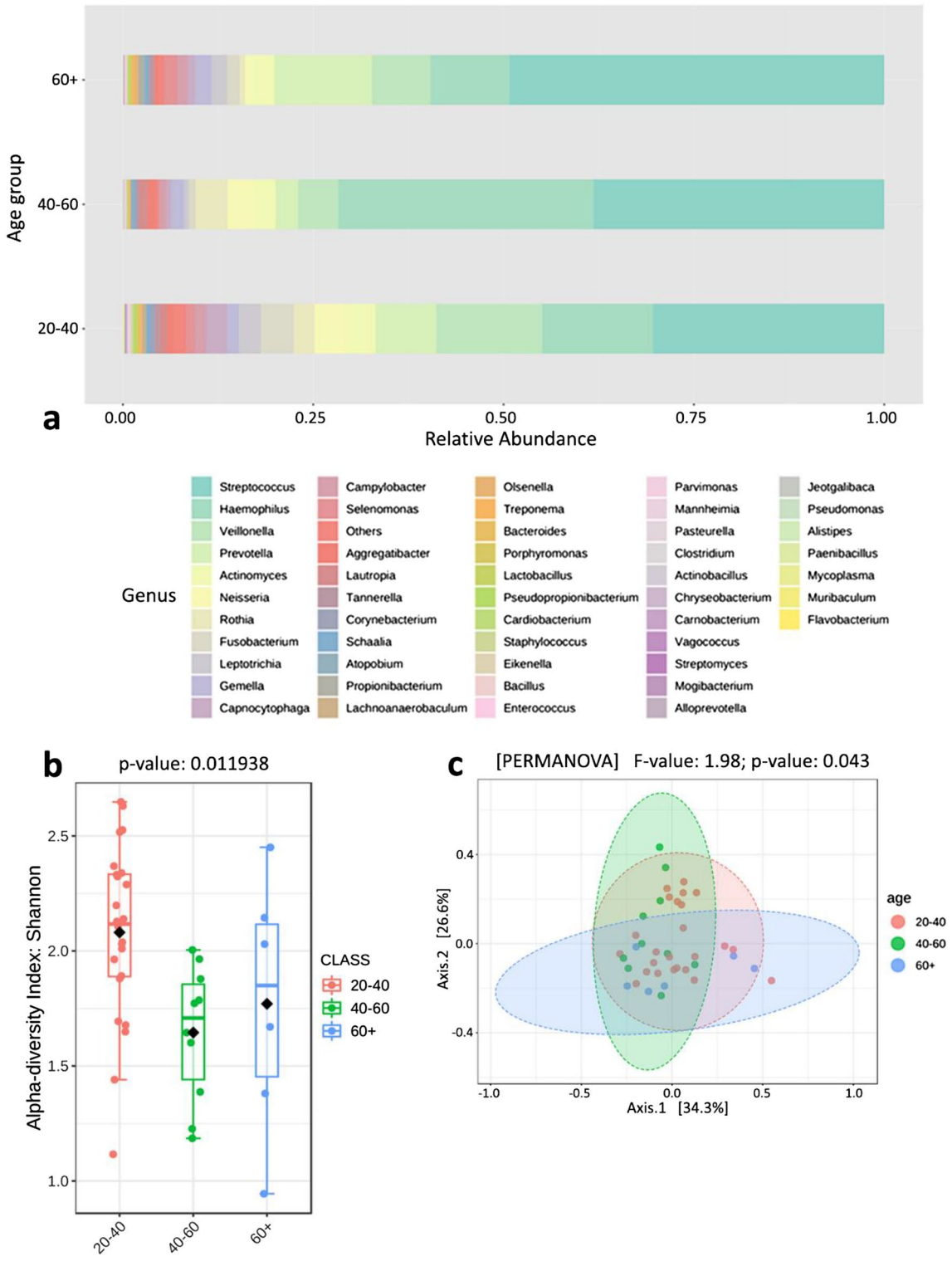

Fig. 1 Genus-level comparison of bacterial profiles in supragingival plaque samples from individuals aged 20–40, 40–60 and 60+ years. **a** Stacked bar plots of the taxonomic classification (percentage abundance), combining samples into three groups. 50 most abundant genera are shown; **b** alpha-diversity analysis, using Shannon diversity index, *T* test/ANOVA, $p = 0.011938$; **c** principal coordinate analysis (PCoA) derived from Bray–Curtis distance among samples of three groups (PERMANOVA, $p = 0.043$). Percentage in square brackets expresses the per cent of variation

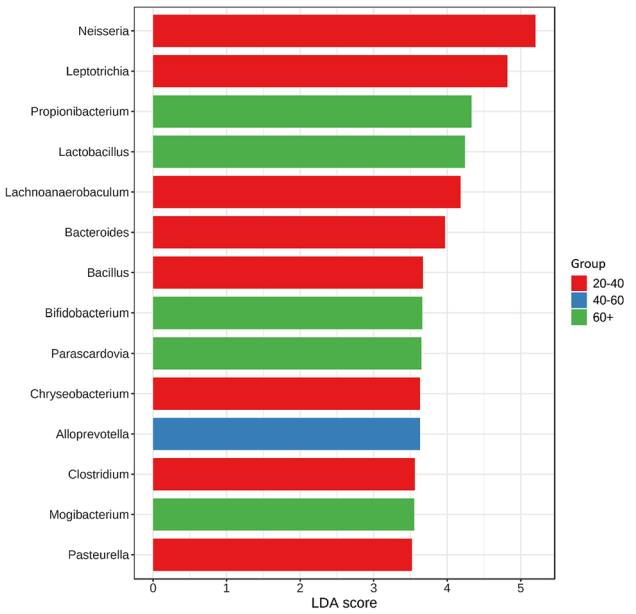

Fig. 2 Linear discriminant analysis (LDA) combined with effect size measurements (LEfSe) identified genera that enabled discrimination between bacterial communities of supragingival plaque microbiome samples from age groups 20–40, 40–60 and 60+ years. False discovery rate (FDR)-adjusted *p* value cutoff: 0.05; logarithmic LDA score ≥ 3.5

Neisseria, Prevotella, Veillonella, Rothia, Actinomyces, Fusobacterium, Gemella, Lautropia, Aggregatibacter and other. Alpha-diversity analysis (*T* test/ANOVA with Shannon diversity index) did not show a statistically significant difference between the three age groups ($p > 0.05$). However, a slight tendency of group 40–60 having the highest taxonomic diversity was observed (Fig. 5b). Beta-diversity analysis of microbial genera composition failed to demonstrate a statistically significant difference between the age groups 20–40, 40–60 and 60+ (PERMANOVA, F value = 0.63, $p > 0.05$) (Fig. 5c). Additionally, linear discriminant analysis effect size (LEfSe) algorithm (*p* value cutoff: 0.05, LDA score: 3.5) did not identify any significant taxonomic features of the age groups.

Taxonomic analysis of bacterial communities in buccal mucosa samples at the species level

A similar scene was observed analysing taxonomic composition of buccal mucosa samples at the species level. Overall composition demonstrated typical oral bacteria (Fig. 6a): *Streptococcus mitis, Streptococcus pneumoniae, Haemophilus parainfluenzae, Streptococcus oris, Veillonella parvula, Neisseria mucosa, Prevotella melaninogenica, Streptococcus gwangjuense, Streptococcus gordonii, Neisseria subflava* and other. This composition, however, also varied slightly in comparison to bacterial species of supragingival plaque samples (Fig. 3a). Both alpha- (*T* test/ NOVA with Shannon diversity index) and beta-diversity (PERMANOVA, F value = 0.59, $p > 0.05$) analyses did not show statistically significant differences between the three age groups (Fig. 6b, c). Similarly, linear discriminant analysis effect size (LEfSe) algorithm (*p* value cutoff: 0.05, LDA score: 3.5) did not identify any distinctive taxonomic features.

Comparing species-level taxonomic profiles of supragingival plaque samples and buccal mucosa samples

Supragingival plaque and buccal mucosa samples showed very similar bacterial profiles with a minor difference between the two groups (Fig. 7a). The most abundant bacterial species in both groups appeared to be *Streptococcus mitis, Haemophilus parainfluenzae, Veillonella parvula, Streptococcus pneumoniae, Streptococcus oralis, Prevotella melaninogenica, Neisseria mucosa, Streptococcus gwangjuense, Haemophilus haemolyticus* and *Fusobacterium nucleatum*, followed by other less abundant species. Although alpha-diversity analysis (*T* test/ANOVA with Shannon diversity index) did not identify a statistically significant diversity difference between the two groups ($p > 0.05$) (Fig. 7b), beta-diversity analysis in the form of PCoA plot (PERMANOVA, F value = 2.14, $p = 0.038$) showed a significant difference between the bacterial composition of the groups (Fig. 7c). We further applied linear discriminant analysis effect size (LEfSe) algorithm (*p* value cutoff: 0.05, LDA score: 3.5) to study deeper the distinctive features of the two groups. On genus level, four distinctive elements of the two groups were identified: *Treponema* for the buccal mucosa sample group and *Lactobacillus, Bacillus* and *Bacteroides* for the supragingival plaque sample group. At the species level, multiple significant distinctive elements were identified within the given parameters. Supragingival plaque samples group was characterized by a significant prevalence of *Streptococcus sanguinis, Streptococcus* sp. *ChDC B345 and Capnocytophaga* sp. *ChDC OS43*. Buccal mucosa sample group, on the other hand, demonstrated 12 bacterial species with statistically significant prevalence: *Neisseria subflava, Streptococcus gwangjuense, Streptococcus sp. 116 D4, Veillonella atypica, Streptococcus sp. oral taxon 064, Streptococcus sp. Oral taxon 431, Fusobacterium pseudoperiodonticum, Prevotella oris, Prevotella denticola, Treponema sp. OMZ 804, Capnocytophaga sp. FDAARGOS 737* and *Gemella sanguinis*.

Taxonomic analysis of supragingival plaque bacterial communities on genus level with respect to individual's sex and smoking status.

Comparing male and female supragingival plaque samples, no significant difference in bacterial genera was observed ($p = 0.72$). (Fig. 8) Beta diversity also did not

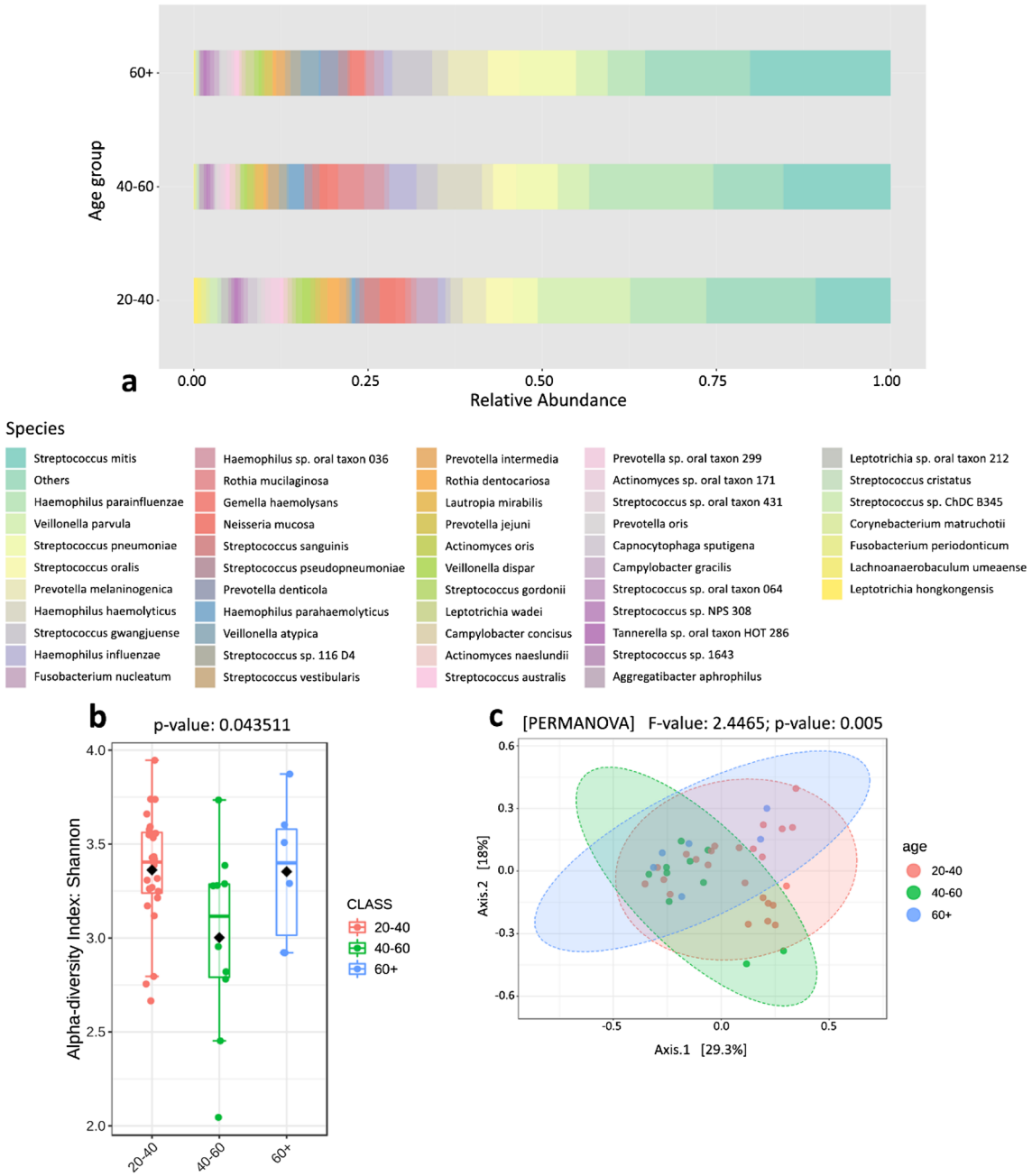

Fig. 3 Species-level comparison of bacterial profiles in supragingival plaque samples from individuals aged 20–40, 40–60 and 60+ years: **a** Stacked bar plots of the taxonomic classification (percentage abundance), combining samples into three groups. 50 most abundant genera are shown; **b** alpha-diversity analysis, using Shannon diversity index, *T* test/ANOVA, $p = 0.043511$; **c** principal coordinate analysis (PCoA) derived from Bray–Curtis distance among samples of three groups (PERMANOVA, $p = 0.005$). Percentage in square brackets expresses the per cent of variation

show any significant difference in microbial composition of sample groups (PERMANOVA, F value = 0.91, $p = 0.43$). The same situation was observed between smoking and non-smoking individual samples—no significant alpha- ($p > 0.56$) or beta- (PERMANOVA, F value = 0.56, $p = 0.76$) diversity difference was found.

Discussion

The goal of this study was to outline the oral microbiome of a general population in Latvia and to identify possible changes appearing within it with age. We also attempted

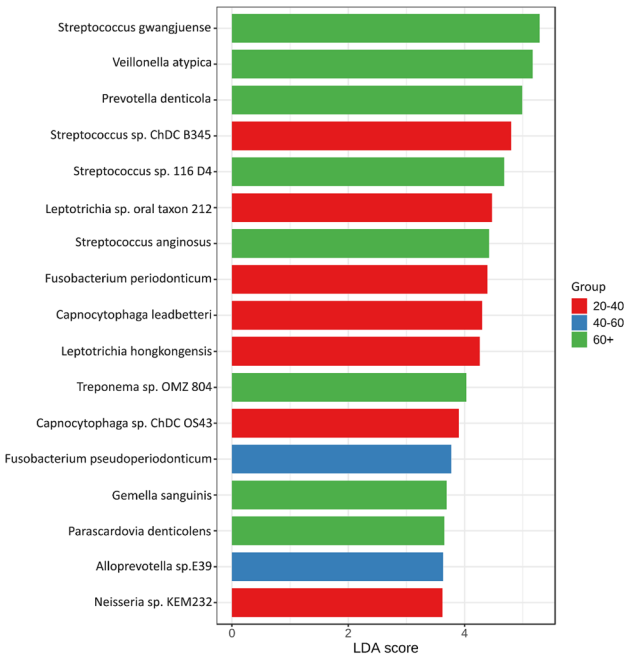

Fig. 4 Linear discriminant analysis (LDA) combined with effect size measurements (LEfSe) identified species enabling discrimination between bacterial communities of supragingival plaque microbiome samples from age groups 20–40, 40–60 and 60+ years. False discovery rate (FDR)-adjusted *p* value cutoff: 0.05; logarithmic LDA score ≥ 3.5

to explore the theory of oral biogeographical locations and evaluate how age influences microbial composition of two oral sites: supragingival plaque and buccal mucosa. Overall, characteristic taxonomic patterns of human oral microbiome were identified with most prevalent species corresponding to typical oral inhabitants (Figs. 3a and 6a) (Aas et al. 2005; Segata et al. 2012; Human Microbiome Project Consortium 2012). Supragingival plaque analysis exhibited statistically significant differences in bacterial composition across the three age groups on both genus and species levels, while buccal mucosa bacterial composition did not differ significantly between age groups. Furthermore, within supragingival plaque samples, several opportunistic pathogens were identified having different prevalence with regard to age groups. Supragingival plaque samples and buccal mucosa samples showed a significant difference in bacterial composition (Fig. 7c); however, no statistically significant difference in bacterial alpha diversity was identified between these two oral locations (Fig. 7b).

Within our study, supragingival plaque samples displayed distinct differences in bacterial composition across the three age groups. Both genus-level (Fig. 1b) and species-level (Fig. 3b) analyses followed similar tendency of middle-aged adults (40–60 years) having the least diverse composition of bacterial communities, whereas adults aged 20–40 years and senior adults (60 + years) had a significantly higher bacterial diversity. These results are largely in agreement with a study by Willis et al. (2022), who identified the 40–50 age group to have the most homogenous oral microbiome composition, whereas age groups 60+ had the most variable composition (Willis et al. 2022). In this study, age group 20–40 years was characterized by the most diverse bacterial genera. Unsurprisingly, species-level analysis also identified a high bacterial diversity, which, as in the case of genus level, experienced a decline within the age group 40–60 years. However, its rise within the age group 60+ is intriguing. On genus level, it rises above the level of the 40–60 group, but does not reach the level of the group 20–40. Species-level analysis, on the other hand, displays a higher rise of bacterial diversity in the group 60+, almost reaching the level of group 20–40 years.

Multiple studies nowadays are focusing on human microbiome diversity in various health conditions and disease states (He et al. 2015; Mosca et al. 2016; Pickard et al. 2017; Manor et al. 2020). Diverse microbiome is often regarded as healthy on the basis of ecological idea that high diversity promotes competition between microbial species, which can be beneficial to the host. However, the cooperation between species may reduce the stability of microbial ecosystem, making it fragile and susceptible towards diseases associated with dysbiosis (Coyte et al. 2015; Frost et al. 2021). Taken together, it is not exactly clear how to interpret microbiome alpha-diversity data and to what extent it reflects the states of health (Johnson and Burnet 2016). For instance, in a study published by Simpson et al., the link between oral microbiome diversity and adolescent anxiety and depression symptoms was explored (Simpson et al. 2020). The findings indicated that although oral microbial diversity levels may remain unchanged, microbiome composition may shift to a state that can be linked with anxiety and depression symptoms in the adolescent population. Same pattern may apply to the case with age-associated oral microbial composition, although we see similar levels of species alpha diversity between group 60+ and group 20–40, beta-diversity analyses of both genus and species levels of these groups separated them in statistically distinct clusters (Figs. 1c and 3c). This indicated that although the diversity rises again after 60+ years, it happens on account of other bacterial species than in the age group 20–40 years.

Exploring changes in supragingival plaque bacterial genera abundance within different age groups, we discovered eight genera that were differentially abundant within the age group 20–40 years, one genus specifically characteristic to the 40–60 age group and five genera whose abundance increased after the age of 60 years (Fig. 2). The most distinctive genus of the group 20–40 years was found to be *Neisseria*, which is an important commensal and one of

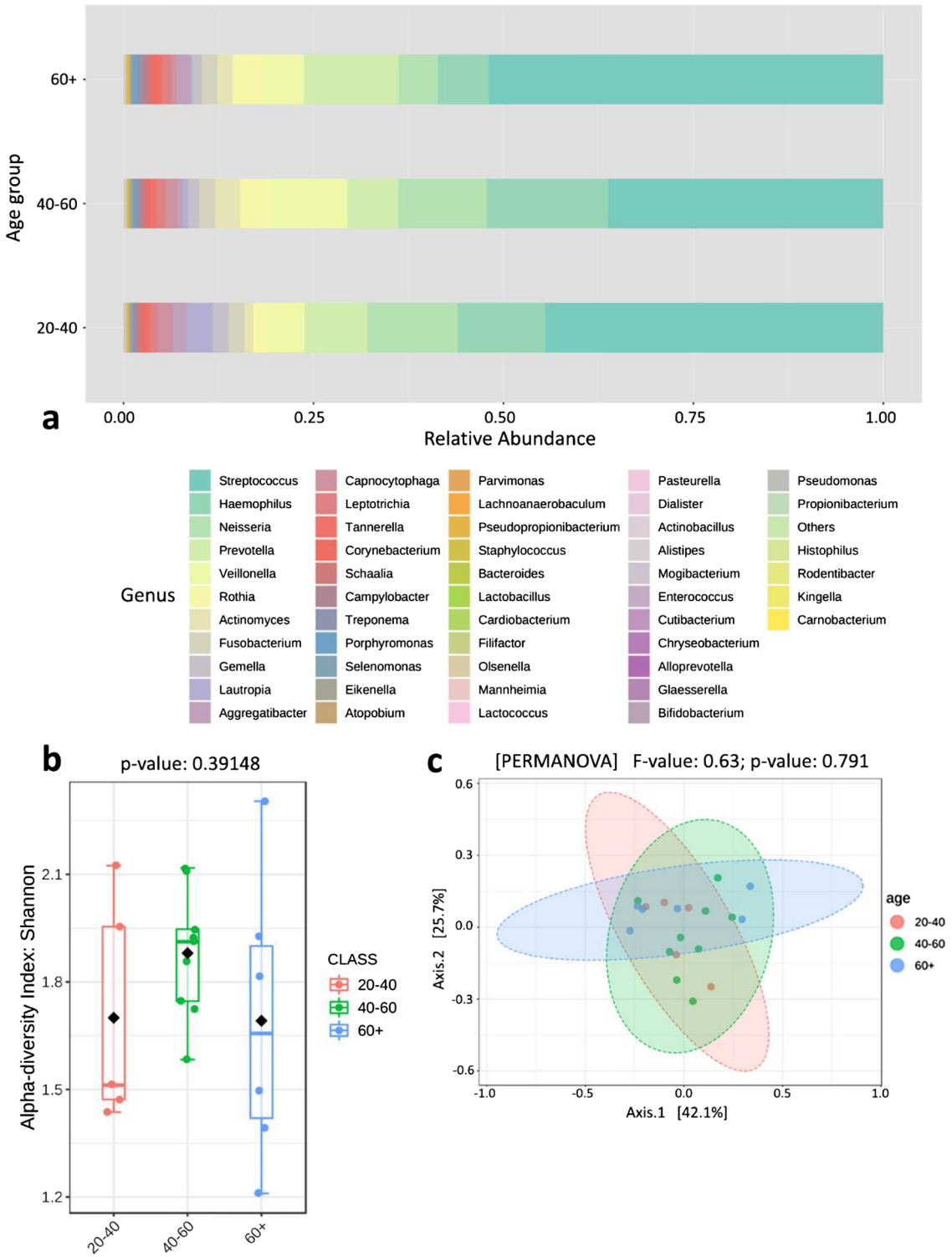

Fig. 5 Genus-level comparison of bacterial profiles in buccal mucosa samples from individuals aged 20–40, 40–60 and 60+ years: **a** Stacked bar plots of the taxonomic classification (percentage abundance), combining samples into three groups. 50 most abundant genera are shown; **b** alpha-diversity analysis, using Shannon diversity index, *T* test/ANOVA, $p=0.39148$; **c** principal coordinate analysis (PCoA) derived from Bray–Curtis distance among samples of three groups (PERMANOVA, $p=0.791$). Percentage in square brackets expresses the per cent of variation

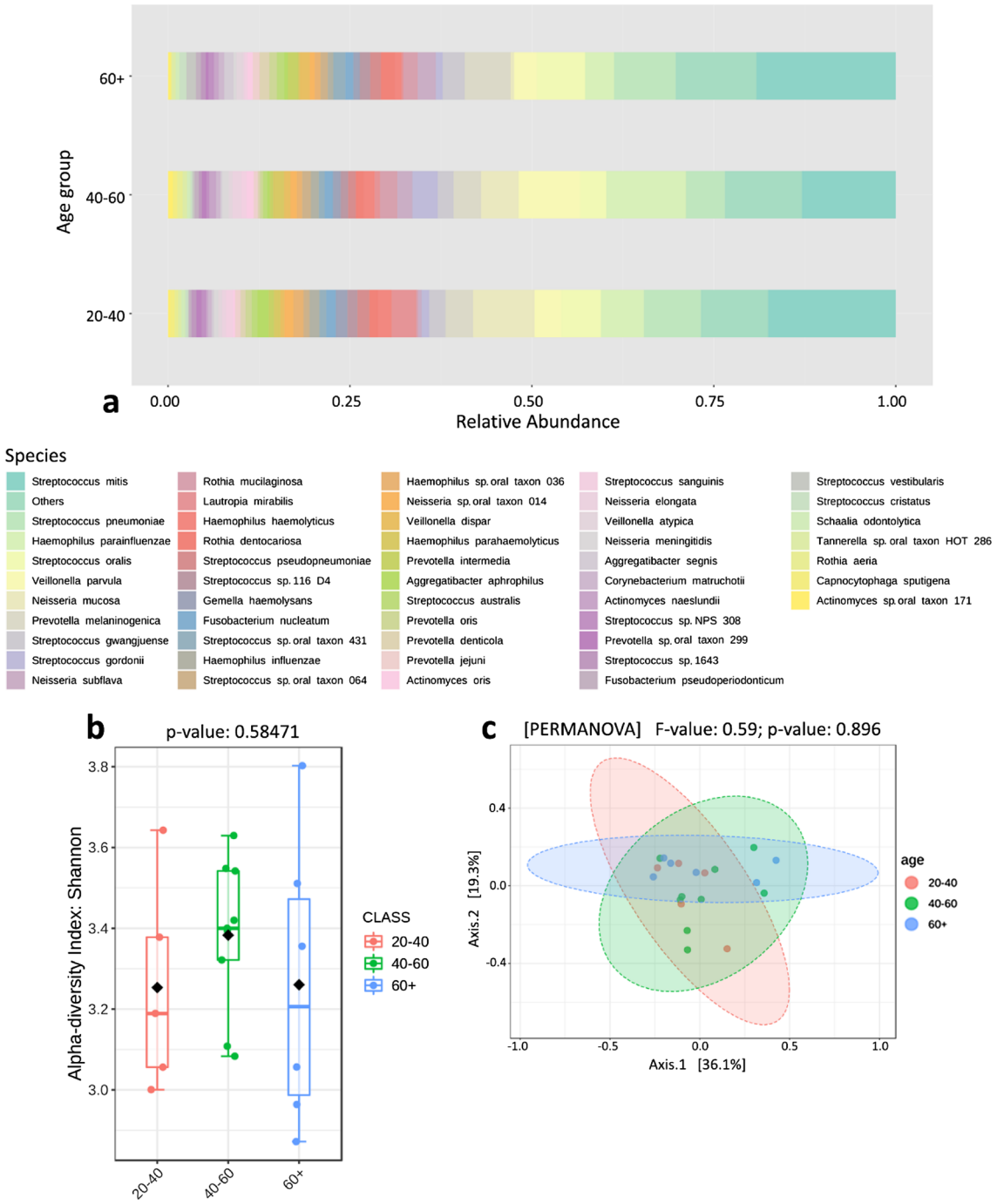

Fig. 6 Species-level comparison of bacterial profiles in buccal mucosa samples from individuals aged 20–40, 40–60 and 60+ years: **a** stacked bar plots of the taxonomic classification (percentage abundance), combining samples into three groups. 50 most abundant genera are shown; **b** alpha-diversity analysis, using Shannon diversity index, *T* test/ANOVA, $p = 0.58471$; **c** principal coordinate analysis (PCoA) derived from Bray–Curtis distance among samples of three groups (PERMANOVA, $p = 0.896$). Percentage in square brackets expresses the per cent of variation

the most prevalent inhabitants of oral microbiota (Sharma et al. 2018; Dorey et al. 2019). A number of studies have emphasized the role of oral non-pathogenic *Neisseria* in local and systemic health. This includes its potential to prevent opportunistic pathogens from colonizing oral and nasal sites and its role in host immune responses (Dorey et al. 2019; Demirci 2021). During the SARS-CoV-2 pandemic, *Neisseria* genus decline in COVID-19 patients in comparison to healthy controls was documented by a number of studies (Wu et al. 2021a; Ma et al. 2021; Iebba et al.

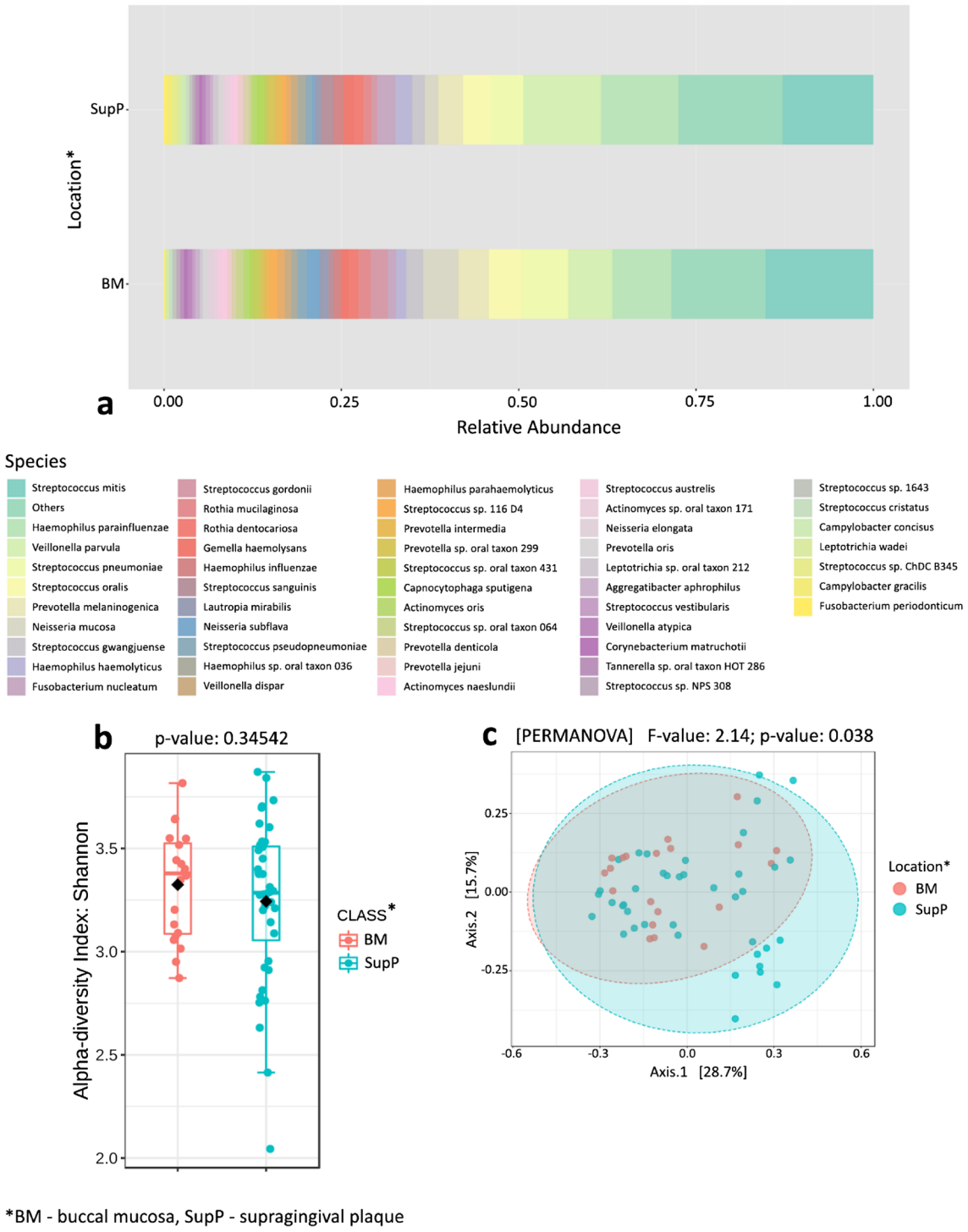

Fig. 7 Species-level comparison of bacterial profiles between supragingival plaque samples and buccal mucosa samples: **a** stacked bar plots of the taxonomic classification (percentage abundance), combining samples into two groups. 50 most abundant genera are shown; **b** alpha-diversity analysis, using Shannon diversity index, *T* test/ANOVA, $p = 0.34542$; **c** principal coordinate analysis (PCoA) derived from Bray–Curtis distance among samples of three groups (PERMANOVA, $p = 0.038$). Percentage in square brackets expresses the per cent of variation

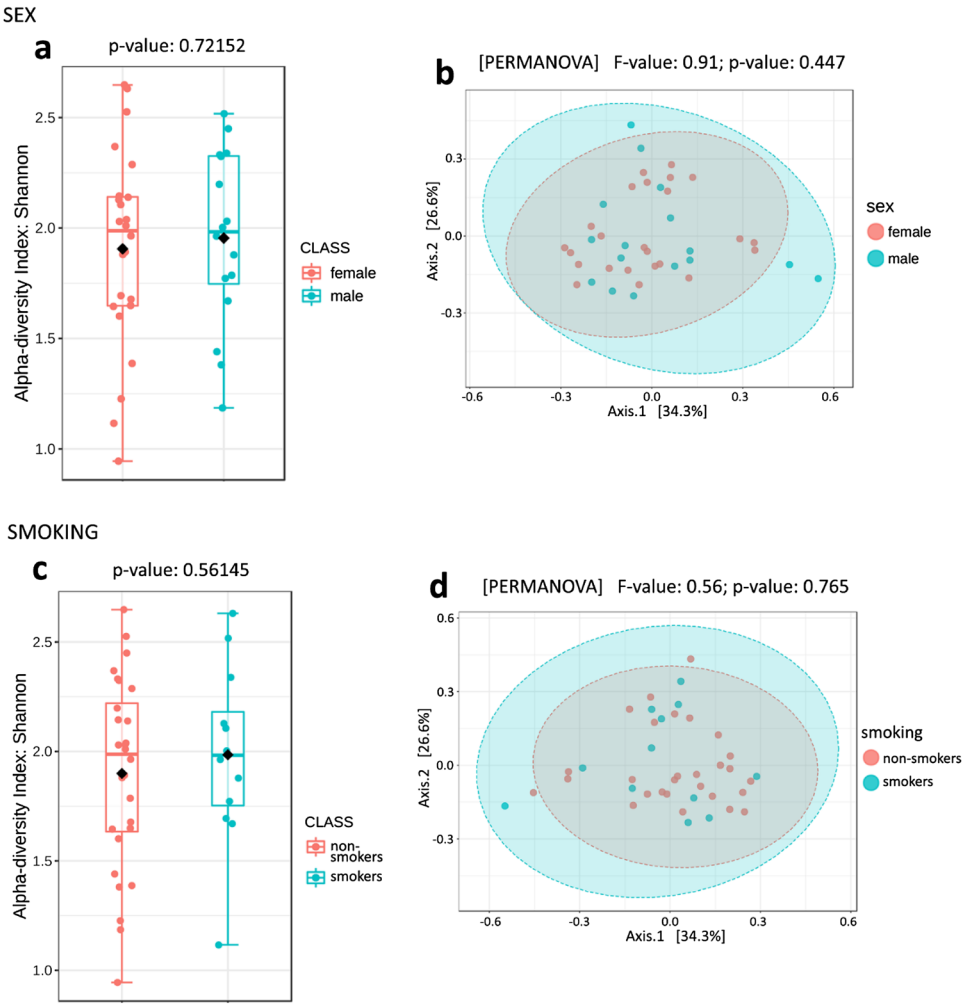

Fig. 8 Genus-level comparison of bacterial profiles from supragingival plaque samples of individuals depending on their sex and smoking status: **a** alpha-diversity analysis of female vs male bacterial communities (Shannon diversity index, $p=0.72152$), **b** principal coordinate analysis (PCoA), (Bray–Curtis, PERMANOVA, $p=0.477$), **c** alpha-diversity analysis of non-smoker vs smoker bacterial communities (Shannon diversity index, $p=0.56145$), **d** PCoA (Bray–Curtis, PERMANOVA, $p=0.765$)

2021). Furthermore, Castilhos et al. reported an increase in COVID-19 mortality rates that could be induced by *Neisseria* decline in oropharyngeal microbiome (de Castilhos et al. 2021). The fact that within our study, genus *Neisseria* was strongly associated with the group 20–40 years, meaning that its prevalence decreased after the age of 40 years, raises a question whether its decline could be linked to age-related health changes. Addressing this question in further studies would be necessary to clarify it.

Among genera, differentially abundant in group 60+ years, the second most significant genus was *Lactobacillus* (Fig. 2). Although it is a common healthy oral microbiota, in a state of dysbiosis it is also considered to be involved in pathological processes both locally and systemically. Within the oral cavity, *Lactobacillus* is a common causal agent of dental caries, metabolizing carbohydrates into organic acids (Takahashi 2015). Recently, several studies have focused on oral microbiome shifts as a possible contributing factor to the formation of neurodegenerative diseases, such as Alzheimer's disease and Parkinson disease (Mihaila et al. 2019; Sureda et al. 2020; Wu et al. 2021b; Jo et al. 2022). A study by We et al. has documented a significant increase of oral *Lactobacillus* genus in Alzheimer's disease patients, while Dragos et al.'s study revealed increase in the abundance of oral *Lactobacillus* in patients suffering from Parkinson's disease (Mihaila et al. 2019; Wu et al. 2021b). While none of our study's participants have mentioned neurodegenerative disorders as their health condition, it is known that development of these diseases begins 10–20 years prior to their clinical manifestation (Sheinerman and Umansky 2013). Chances are that oral microbiome shifts, indicating age-related diseases, might appear prior to any clinical symptoms. More studies, however, are needed to test this hypothesis.

Analysing species-level differential results for three age groups of supragingival plaque samples (Fig. 4), several intriguing observations can be made. Group 60+, within this analysis, is characterized by eight bacterial species. Among common oral commensals such as *Veillonella atypica* and *Prevotella denticola*, it is notable that we see also the presence of *Streptococcus anginosus* and *Gemella sanguinis*, which are considered to be important opportunistic

pathogens, involved in a variety of pathological conditions. Furthermore, the abundance of both bacteria has a tendency to increase gradually with age, as shown by univariable analysis (Fig. 9). *Streptococcus anginosus*, together with *Streptococcus intermedius* and *Streptococcus constellatus,* belongs to the *Streptococcus anginosus* group, which is associated with overall dysbiosis in oral cavity and an increasing number of reports of systemic life-threatening conditions such as cardiovascular diseases (pericarditis, endocarditis), pulmonary disorders (pneumonia, pleural empyema) and head and neck disorders (Lemierre's syndrome, orofacial, brain and intracranial abscesses) (Pilarczyk-Zurek et al. 2022). *Gemella sanguinis* is another member of human oral microbiome which may occasionally cause systemic infections, with endocarditis being the most common among them (Gundre et al. 2011; Yang and Tsai 2014; García López and Martín-Galiano 2020).

Evaluating distinctively present opportunistic pathogens in different age groups, however, possesses challenges. The most fundamental challenge is related to the thin line between commensal and opportunistic bacteria within the human microbiome. Manifestation of bacterial pathogenesis of any opportunistic pathogen is a complex multifactorial process, which involves overall systemic

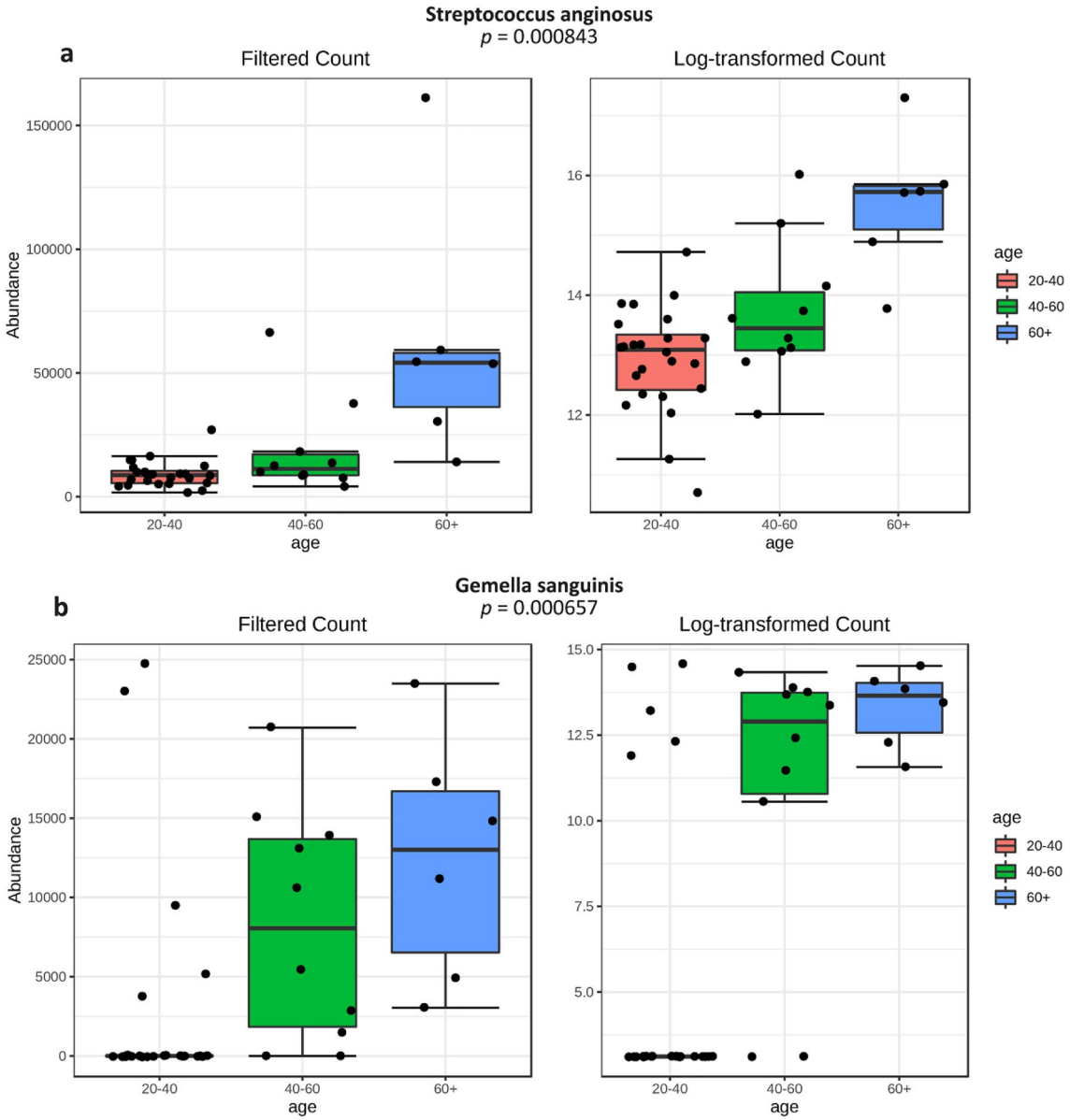

Fig. 9 Univariable analysis of two opportunistic pathogenic species, identified by linear discriminant analysis (LDA) combined with effect size measurements (LEfSe) to be significant features of sample group 60+: **a** *Streptococcus anginosus* ($p = 0.000843$), **b** *Gemella sanguinis* ($p = 0.000657$)

health conditions of the patient as well as microbiome stability status, leading to the fact that almost any commensal bacteria can potentially become opportunistic under specific circumstances. For instance, *Streptococcus mitis* is a predominant commensal inhabiting the human oropharynx as well as gastrointestinal tract, skin and female genital tract (Kutlu et al. 2008; Mitchell 2011). It was also found to be one of the most abundant bacteria within all sample groups of the present study (Figs. 3a and 6a). Under normal conditions, it has important physiological functions in the oral cavity such as inflammatory response modulation and participation in wound healing (Engen et al. 2017). Nonetheless, under the conditions of a vulnerable immune system, *S. mitis* might relocate from its niche and apply its effective colonization strategies as virulence factors causing dangerous systemic health disorders such as bacteraemia, septicaemia and infective endocarditis (Mitchell 2011; Eberhard et al. 2017). Taken together, these facts indicate that the host immune system and oral microbiome composition are interconnected entities that influence each other bidirectionally. Ageing is considered to be one of perturbation factors on the basis of which opportunistic pathogens manifest their virulence (Brown et al. 2012). Hence, increased abundance of opportunistic pathogenic bacteria within the ageing host might serve as a risk factor for disease development. Nevertheless, evaluation of microbiome state on the basis of opportunistic pathogen presence solely might not be enough to drive meaningful conclusions and adjacent health-related factors must be taken into account.

We further attempted to investigate age-related microbial differences in buccal mucosa samples, which is a separate oral biogeographical location with its distinct physiological characteristics. It has been determined that various oral niches such as buccal mucosa, saliva, tongue, teeth surfaces, gums, palate and both subgingival and supragingival plaque, as well as the throat and tonsils, are similar in terms of microbial composition, but also possess unique attributes (Segata et al. 2012; Human Microbiome Project Consortium 2012; Xu et al. 2015). In contrast to supragingival biofilm, which is known to contain a mixture of aerobes and facultative anaerobes, buccal mucosa bacterial composition mostly comprises aerobic bacteria (Arweiler and Netuschil 2016). Although existing literature underlines the divergence of bacterial profiles between various oral sites, a number of studies emphasize a coherence between microbiomes of different oral niches, concluding that microbial shifts within one niche have an echoing effect on the niches around (Wei et al. 2019; Liu et al. 2020). Despite these expectations, analysis of bacterial communities within buccal mucosa samples of the present study, in contrast to supragingival plaque samples, did not show any statistically significant alpha- or beta-diversity differences between the three age groups (Figs. 5b, c and 6b, c). One of the possible explanations could be a small number of buccal mucosa samples involved in the study.

Comparing the bacterial composition of supragingival plaque to buccal mucosa disregarding age, however, few significant observations were made. Supragingival plaque microbiome is often defined as a more diverse bacterial community in comparison with the microbiome of buccal mucosa (Xu et al. 2015). This is logical considering physiological differences of these two niches. Supragingival plaque is formed on a non-shedding tooth surface which favours biofilm formation, while buccal mucosa's epithelial cells constantly shed, and hence less time is available for complex biofilm development (Mager et al. 2003; Besemer et al. 2012). Within our study, however, no significant bacterial alpha-diversity difference was detected between supragingival plaque and buccal mucosa samples (Fig. 7b). On the other hand, beta-diversity analysis in the form of principal coordinate analysis (PCoA) separated both sample groups into two statistically distinct clusters (Fig. 7c). This result is in accordance with the theory of distinct oral biogeographical locations, postulating significant difference between the microbial patterns of the two oral sites.

Evaluation of these distinct patterns, however, brings us back to a variety of challenges involved in oral microbiome data interpretation. Our notion about human microbiome, in general, and oral microbiome, in particular, is far from being complete. Multiple studies have highlighted the existence of distinct microbial subgroups even within the same populations (Filippis et al. 2014; Zaura et al. 2017; Willis et al. 2018; Willis and Gabaldón 2020). This illustrates the complexity of oral microbiome structure, taken together with countless factors that influence it. For example, genus *Corynebacterium*, which was identified to be a characteristic element of both supra- and subgingival plaques by other studies (Segata et al. 2012; Human Microbiome Project Consortium 2012; Mark Welch et al. 2019), did not perform as a distinctive element between supragingival plaque and buccal mucosa within our study (Fig. 10a; Fig. S2, Supplementary Material). Nevertheless, this might be only a surface difference, as there is evidence of microbial taxa variability on the background of stable metabolic pathways within a healthy population (Human Microbiome Project Consortium 2012). Metabolomic analyses should be implemented in future studies to address this issue.

Within supragingival plaque samples, genus *Lactobacillus* appeared to be distinctively prevalent in comparison to the buccal mucosa (Fig. 10a). This is notable, as same genus was found as a distinctive feature of the age group 60+ within supragingival plaque samples (Fig. 2). On the contrary, *Gemella sanguinis*, an opportunistic pathogen significantly more prevalent within the 60+ age group of supragingival plaque samples (Fig. 4), appeared to be a

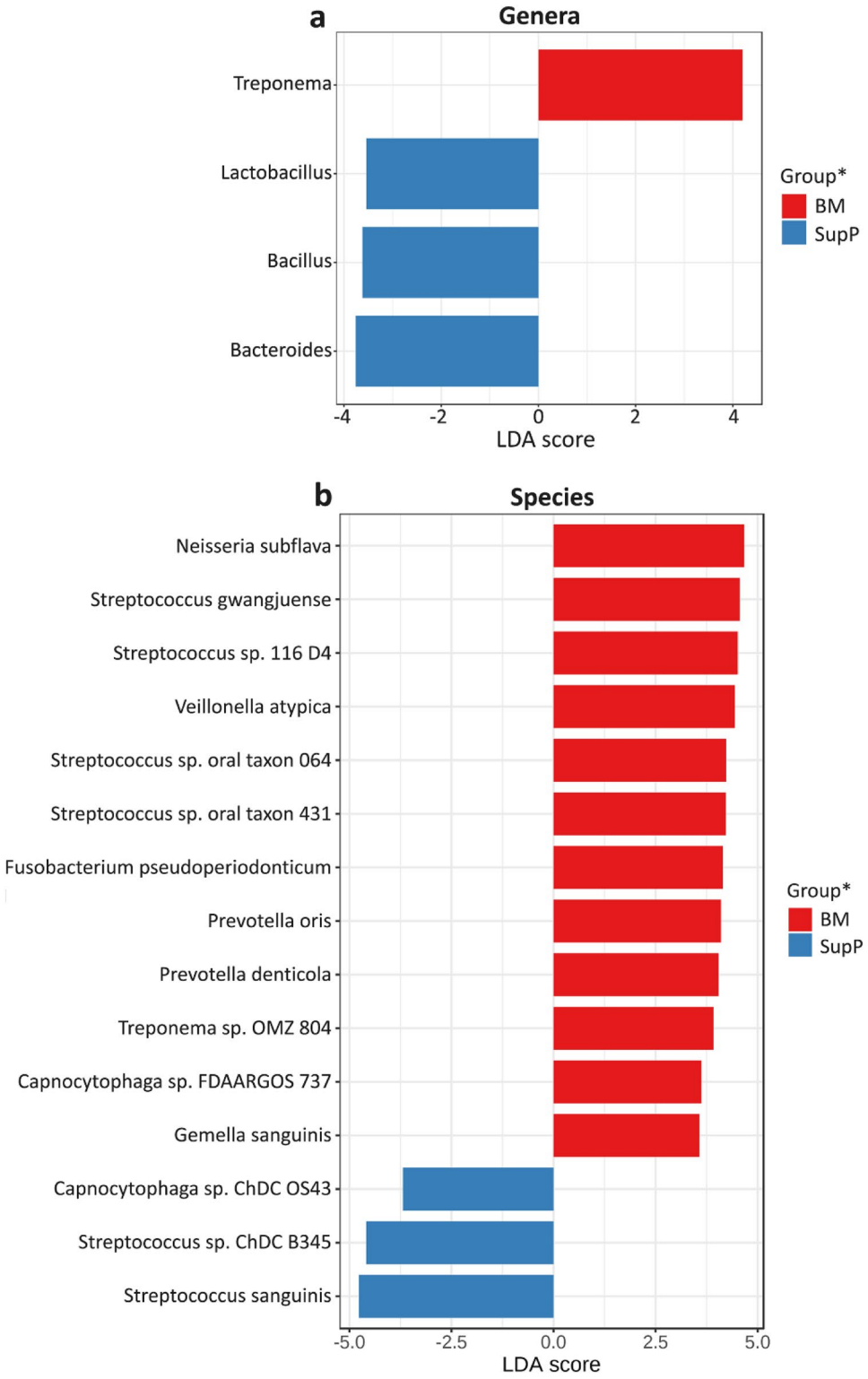

Fig. 10 Linear discriminant analysis (LDA) combined with effect size measurements (LEfSe) identified taxons that enabled discrimination between bacterial communities of supragingival plaque microbiome samples and buccal mucosa microbiome samples at genus level (**a**) and species level (**b**). False discovery rate (FDR)-adjusted *p* value cutoff: 0.05; logarithmic LDA score ≥ 3.5

distinctive feature of buccal mucosa (Fig. 10b), although it did not show age-related prevalence difference within the buccal mucosa samples. Taken together, these observations indicate that age-related findings might be influenced by the oral biogeographical location.

Further, we have compared supragingival plaque bacterial patterns of individuals based on their sex (male vs female). It is known that some periodontitis-associated bacterial species are influenced by human sex hormone levels, which gets secreted into the saliva and in this way enters the oral

cavity (Cornejo Ulloa et al. 2021). In our study, however, no significant bacterial abundance or composition difference was observed between the male and female samples (Fig. 8a, b). A larger number of samples could be needed to address this question.

Finally, we have attempted to compare supragingival plaque microbiome of individuals based on their smoking status. In recent years, numerous studies have been published exploring the effect of different types of smoking on oral microbiome in general and supragingival plaque microbiome in particular (Wu et al. 2016; Jia et al. 2021; Al-Marzooq et al. 2022). Results of these studies vary, nevertheless the majority of them emphasize the significant difference between smokers and non-smokers oral microbiome patterns. Despite these findings, within our samples no significant difference in alpha and beta diversity between smokers and non-smokers group was noted (Fig. 8c, d). Again, a small number of samples, involved in this study, might be the reason of these results.

Conclusion

In this study, we explored oral microbiome variations in the general population with regard to ageing. We found that adults (20–40 years) had the highest bacterial diversity in supragingival plaque, which experienced a decline during the 40–60 age period and then rose again after the age of 60 years. All three age groups exhibited distinct bacterial communities. The important oral commensal *Neisseria* had declined after the age of 40 years, while genus *Lactobacillus*, suspected as contributing to neurodegenerative disorders, increased after the age of 60 years. Additionally, the prevalence of two well-documented opportunistic pathogens *Streptococcus anginosus* and *Gemella sanguinis* gradually rose with age within our samples. Furthermore, we found age-related oral microbiome variations to be niche dependent, as buccal mucosa microbiota did not show significant age-related changes. However, supragingival plaque and buccal mucosa samples significantly differed in bacterial composition, confirming the theory of oral biogeographical locations.

Nevertheless, there are certain limitations to this study. Firstly, a narrow range of samples and limited number of documented confounding variables restrict the possibilities to examine oral microbiome fluctuations that are linked to specific health conditions. Secondly, broad in-group age spectrum may limit the possibility to draw conclusions of age comparison. Important oral microbiome trends, however, were noticeable even at the existing scale. Lastly, the methodological approach, implemented in this research, restricts the study to a taxonomic level of analysis – a broader insights into the oral microbiome ecosystem could be gained incorporating functional analysis of metabolic pathways, which should be considered in future studies.

The results of this study are relevant to the scientific community, as they demonstrate that oral microbiome transforms across age following certain patterns which may be related to systemic changes in various locations of the body. It is possible that earlier in life, we build the foundation of bacterial communities in oral cavity that gradually develop into resilient ecosystem and influence our health further in life, providing us either a healthy life span or age-related health problems. Although oral dysbiosis seems to contribute to disease formation in the oral cavity as well as in distant body sites, there are still many questions that remain unanswered and many challenges remain ahead. Accumulating data on the subject of oral microbiome and its changes across time will support the progress of the research area, dedicated to a healthy life span.

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Author contributions All authors contributed to the design and execution of experiments, interpretation of data, preparation of figures and writing the manuscript.

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Data availability The metagenomes data are available in the Sequence Read Archive hosted at the NCBI under the BioProject accession code PRJNA931979.

Declarations

Conflict of interest The authors declare that they have no conflicts of interest with the contents of this article.

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