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Oral microbiome in Proliferative Verrucous Leukoplakia exhibits loss of diversity and enrichment of pathogens

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ABSTRACT

Objectives: Oral microbiome plays an important role in oral diseases. Among them, proliferative verrucous leucoplakia (PVL) is an uncommon form of progressive multifocal leukoplakia with a worryingly rate of malignant transformation. Here, we aimed to characterize the oral microbiome of PVL patients and compare it with those of healthy controls.

Material and methods: Oral biopsies from ten PVL patients and five healthy individuals were obtained and used to compare their microbial communities. The sequence of the V3-V4 region of 16S rRNA gene was used as the taxonomic basis to estimate and analyze the composition and diversity of bacterial populations present in the samples.

Results: Our results show that the oral microbial composition and diversity are significantly different among PVL patients and healthy donors. The average number of observed operational taxonomic units (OTUs) was higher for healthy donors than for PVL, proving a loss of diversity in PVL. Several OTUs were found to be more abundant in either group. Among those that were significantly enriched in PVL patients, potential protumorigenic pathogens like *Oribacterium* sp. *oral taxon 108*, *Campylobacter jejuni*, uncultured *Eubacterium* sp., *Tannerella*, and *Porphyromonas* were identified.

Conclusion: Oral microbiome dysbiosis was found in patients suffering from PVL. To the best of our knowledge, this is the first study investigating the oral microbiome alterations in PVL and, due to the limited number of participants, additional studies are needed. Oral microbiota-based biomarkers may be helpful in predicting the risks for the development of PVL.

Introduction

Proliferative verrucous leukoplakia (PVL) is an oral potentially malignant disorder which initially manifests as white asymptomatic hyperkeratotic plaques, but in time progresses affecting different and multiple oral mucosa locations with a warty tendency during its

Abbreviations: CagA, cytotoxin associated gene A; CDT, cytolethal distending toxin; CRC, colorectal carcinoma; eHOMD, expanded Human Oral Microbiome Database; FadA, Fusobacterium effecto adhesion A; HCC, hepatocellular carcinoma; HPV, human papillomavirus; IpgD, inositol phosphate phosphatase D; LPS, lipopolysaccharide; MP toxin, metalloproteinase toxin; NGS, next-generation sequencing; OTU, Operative Taxonomical Unit; OSCC, Oral Squamous Cell Carcinoma; PCA, Principal Components Analysis; PVL, proliferative verrucous leucoplakia; RDP, Ribosomal database Project; VirA, virulence gene A; VST, Variance stabilizing transformation.

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evolution [1]. It was first described by Hansen et al. in 1985 and it is characterized by a tremendous treatment resistance, a high rate of recurrences and development of new leukoplakias, and a very high risk of transformation into oral cancer [2,3]. The overall incidence of malignant transformation was first considered around 40% [4–6], but long-term studies have shown transformation rates approaching 70–100% [3,7]. Second primary tumors and field cancerization are also frequent in PVL, with decreasing time intervals between the development of one tumor and the next one over time [8]. The worryingly high rate of malignant transformation is aggravated by the inefficacy of clinical procedures in the long term, rendering scalpel or laser removal insufficient [2,9]. Local block tissue removal, often including nearby teeth, is sometimes the only viable procedure [10]. PVL is preponderant in elderly women, and contrary to common oral leukoplakia, it has not been associated to tobacco-related habits [11,12]. A previous study conducted by our group including 55 PVL patients concluded that patients who develop oral cancers are commonly non-smokers females and those who develop more than one oral squamous cell carcinoma (OSCC) are more likely to develop lesions of the gingiva [13].

In spite of the environmental and genetic risk factors, scientists have realized during the last decades that microorganisms inhabiting the human body take an active role in the maintenance of health and the appearance of diseases. Microbiome studies, motivated by the availability of high-throughput technologies, have exhibited how the disturbance of the microbiota is associated with a great number of human diseases [14]. To date, the vast majority of studies have been performed on the gut, which constitutes the body niche where most of commensal microorganisms reside. As a result, associations between gut microbiota and diseases, either in a positive or in a negative way, have been found, including inflammatory bowel disease, multiple sclerosis, type 1 and 2 diabetes, allergies, asthma, autism, and cancer [15,16]. In particular, altering gut microbiota composition affects the incidence and progression of colorectal carcinoma (CRC) in both genetic and carcinogen-induced models of tumorigenesis [17–19]. Several by-products of the gut microbiota directly target intestinal epithelial cells, mediating oncogenic effects as reported for cytolethal distending toxin (CTD), inositol phosphate phosphatase D (IpgD), virulence gene A (VirA), cytotoxin associated gene A (CagA), Fusobacterium effecto adhesion A (FadA), metalloproteinase toxin (MP toxin), among others, or suppress tumorigenesis, as demonstrated for butyrate, propionate, monophosphoryl lipid A, ferrichrome, pyridoxine and lipopolysaccharide (LPS) [20–22]. Experimental alterations of the gut microbiota have also been observed in other malignancies, including hepatocellular carcinoma (HCC) and breast cancer. Through the portal venous system, the liver is exposed to intestinal bacteria and their by-products, which could cause inflammatory changes, hepatotoxicity and carcinogenesis [23]. In the case of breast cancer, it is hypothesized that the gut microbiota may promote carcinogenesis via its ability to alter the profile of circulating estrogens and phytoestrogens, via its influence on energy metabolism or via antitumor immune function [24,25]. More studies have been published which report a link between gut dysbiosis and other malignancies, but these associations are less well established and require further investigation. Although it has been less studied, oral microbiome dysbiosis could be linked to oral cancer and other oral diseases through direct metabolism of chemical carcinogens and general inflammatory effects [26]. The oral cavity is home for more than 700 microbial species, including commensal and opportunistic bacterium, fungi and viruses, and despite PVL having an unknown etiology, controversy around the possible implication of human papillomavirus (HPV) infections, like HPV 16 infection, in its pathogenesis exists [5,9,27,28]. Moreover, dysbiotic oral microflora has already been associated to chronic periodontal disease [29,30] and chronic bacterial infection might be promoter of oral cancer [31] but there are not studies analysing the influence of oral microbiome in the development and/or progression of PVL. In this study, oral biopsies of PVL patients were used to characterize their microbiome and compare it with that of healthy controls in order to gain insight into the possible role of oral microorganisms in PVL and its malignant transformation to cancer. To the best of our knowledge, this is the first time that alterations in the oral microbiome of PVL patients have been investigated.

Patients and methods

Patients and tissue samples

This study included 10 patients with a clinical diagnosis of PVL and a control group comprised of 5 healthy donors. For the PVL patients, two representative biopsies were taken from the same area of the lesions, including epithelium and the underlying connective tissue between 2017 and 2018. One of each pair of specimens was analyzed with the routine histopathological methods to ensure that each patient met the histopathological criteria to establish the PVL diagnosis provided by Cerero-Lapiedra et al. [32]. Briefly, there can be from simple epithelial hyperkeratosis to verrucous hyperplasia with or without epithelial dysplasia. In addition, the criteria used for diagnosing oral epithelial dysplasia reported by Warnakulasuriya S et al. was followed [33]. It is based on changes to the architecture of the epithelium and those that manifest as cellular atypia. Conventionally, dysplasia is divided into mild (architectural disturbance limited to the lower third of the epithelium, accompanied by minimal cytological atypia), moderate (architectural disturbance extending into the middle third of the epithelium), and severe (more than two thirds of the epithelium shows architectural disturbance with associated cytological atypia). Another biopsy was required in order to rule out the presence of OSCC. The other sample was used for the 16S sequencing. For the control group, samples were obtained from healthy mucosa areas adjacent to the teeth (vestibular fundus). All tissue samples were frozen at −80 °C until their analysis. A previous transcriptomic and epigenetic characterization of the same cohort of patients (see Table 1) has been performed using RNAseq and is available at [34,35].

Library preparation and Illumina sequencing

The sequence of the V3-V4 region of 16S rRNA gene was used as the taxonomic basis to estimate bacterial populations present in the samples, according what stated in Caporaso et al. [36]. DNA concentration was determined in the samples using a fluorimetric method using Quant-IT PicoGreen reagent in a FLUOROSCAN fluorimeter (Thermo Fisher). Afterwards, DNA samples were diluted to 1 ng/ul and 2 ul of each sample were used to amplify the V3-V4 region of 16 S rRNA gene using specific primers for 16S rRNA with the following sequences:

Forward primer: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3′.

Reverse primer: 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3.

Those primers were used as fusion primers respectively linked to CS1 and CS2 sequences (Fluidigm) useful for subsequent barcoding. Positive amplification (22–25 cycles) was evaluated by gel electrophoresis of PCR products which showed a marked and clean band of a size around 460 pb. Therefore, a second PCR of low number of cycles was applied to add the individual barcode to each of the samples, as well as to incorporate Illumina-specific sequences in the amplicon libraries. Individual libraries were analyzed using a Bioanalyzer 2100 (Agilent) to estimate the concentration of the specific PCR products and a pool of samples was made in equimolar amounts. The pool was further cleaned, quantified and the exact concentration of the library was measured by real time PCR, using Illumina specific primers (Kapa Biosystems). Finally, samples were denatured to be seeded onto a Miseq v3 flowcell (Illumina) and run under a 2x300 pair end sequencing procedure (Scientific Park of Madrid, Spain). A total amount of 180.000 pair end reads was obtained as average (range 146.000–213.000). After quality filtering and demultiplexing, fastq files were prepared and submitted to the bioinformatic

Table 1
Clinicopathological information of the PVL patients included in this study. A previous transcriptomic and epigenetic characterization of the same cohort of patients was performed and is available at (25,26).

PVL patients					Clinical and histological characteristics of PVL before developing OSCC				OSCC		
Case	Age	Gender	Tobacco smoker	Follow up of PVL (Years)	Number of oral locations	Oral locations	Percentage of oral mucosa affected with PVL lesions	Histological findings when taken the sample for 16S sequencing	Transformation in OSCC	Number of oral cancers	Location of oral cancers
1	66	Male	No	7	3	Gingiva, Buccal mucosa, Lips	40	Without dysplasia	Yes	2	Buccal mucosa
2	71	Male	No	3	2	Gingiva, Buccal mucosa	40	Moderate dysplasia	No	–	–
3	60	Female	No	19	5	Gingival, Buccal mucosa, Tongue, Floor mouth, lip	75	Without dysplasia	Yes	2	Gingiva
4	86	Male	No	10	4	Gingiva, Buccal mucosa, Floor mouth, Tongue	50	Mild dysplasia	No	–	–
5	73	Female	Yes	3	2	Gingiva, Buccal mucosa	25	Severe dysplasia	Yes	2	Gingiva, Buccal mucosa
6	83	Females	No	10	6	Gingiva, Buccal mucosa, Lips, Tongue, Floor mouth, Palate	90	Mild dysplasia	Yes	1	Gingiva
7	63	Female	No	9	5	Gingiva, Buccal mucosa, Palate, Floor mouth, Tongue	50	Moderate dysplasia	Yes	1	Gingiva
8	70	Male	Yes	15	2	Gingiva, Buccal mucosa	30	Moderate dysplasia	Yes	3	Gingiva
9	80	Female	No	20	5	Gingiva, Buccal mucosa, Palate, Floor mouth, Tongue	50	Moderate dysplasia	No	–	–
10	67	Female	Yes	26	4	Gingiva, Buccal mucosa, Palate, Tongue	65	Severe dysplasia	Yes	1	Gingiva

pipeline.

Bioinformatic data processing and analyses

Quality Analysis and preprocessing: Quality control on Fastq libraries was performed using FastQC [37]. Subsequently, Fastq files were pre-processed using Prinseq-lite-020.4 [38] to eliminate primers and low-quality sequences. Paired-end Fastq files were merged and then converted to fasta format using the QUIIME scripts "join_paired_ends.py" and "convert_fastaqual_fastq.py" available here http://qiime.org/scripts/index.html.

Characterization, quantification and binning of Operative Taxonomical Units (OTUs): Cd-hit-est of CD-HIT [39] was used to group the pre-processed reads of each fasta library into OTU clusters using a sequence similarity cut-off of 0.95. The most representative sequence of each OTU cluster (provided by CD-HIT together with the counts per OTU) was then mapped against the Ribosomal database Project (RDP) release 11 [40] using HS-BLASTN [41] to bin each OTU. Finally, counts and taxonomy annotations for each OTU were extracted from the CD-HIT and HS-BLAST outputs and integrated together using the Worksheet App of the GPRO suite [42].

Differential Distribution: DESeq [43] was used to assess differential distribution of OTUs between the two sample groups (PVL and control). OTU counts were previously normalized also with DESeq by dividing the counts by the size factors and applying variance stabilizing transformation (VST).

Statistical analyses: All statistical analysis performed in this study, including the creation of KRONAs interactive charts [44] per sample and group, were performed using the R scripts implemented in the STATools App of the GPRO suite.

Data availability

Raw data have been deposited at the NCBI SRA archive with

BioProject record PRJNA663437, and BioSample records (SAMN16132437, SAMN16132438, SAMN16132439, SAMN16132440, SAMN16132441, SAMN1613242, SAMN16132443, SAMN16132444, SAMN16132445, SAMN16132446, SAMN16132447, SAMN16132448, SAMN16132449, SAMN16132450, SAMN16132451).

Ethics statement

This study was approved by the Ethics Committee for Human Research of the University of Valencia (Ref. H1523722754549). Informed written consent was obtained from all participants after an explanation of the nature of the study, as approved by the Ethics Committee for Human Research of the University of Valencia.

Results

Participant characteristics and sequencing data summary

This study included a cohort of 10 patients with PVL and a cohort of 5 healthy donors as controls. The clinical profile and clinicopathological information from each of the PVL patients is shown in Table 1. The median patient age was 70.5 years [range: 60–86], 60% were females, and 30% were smokers. The median follow-up of the study was 10 years [range: 3–26]. Two patients did not have dysplasia, two showed mild dysplasia, four moderate dysplasia, and two severe dysplasia. None of the selected PVL cases presented histopathological signs of OSCC at the time of the biopsy. However, it is noteworthy that after 3 years of study, 7 out of 10 PVL cases developed at least one OSCC and 4 of them have developed more than one.

Sequencing of oral samples resulted in a total of 2,735,102 raw sequences. Filtering for sequence quality reduced the number of sequences to 2,472,087, being 2,364,853 (95.6%) assigned to 980 OTUs. Rarefaction curves show that the 15 samples were sequenced to a sufficient depth such that a complete microbiome profile was likely captured for most samples (Fig. 1A). Regarding *Bacteria*, a total of 37 phyla, 53 orders, 94 families, and 630 genera were identified, whereas 7 phyla, 13 orders, 18 families, and 40 genera were identified for *Archaea*.

Microbiome diversity and composition of PVL and healthy oral samples.

An unsupervised principal component analysis (PCA) including all samples was performed in order to group samples according to their microbiome. PCA score plot revealed that microbiome from PVL patients is more homogeneous than that from healthy donors (Fig. 1B). In addition, Mann-Whitney *U* test confirmed that the distributions of both groups is significantly different, being the oral diversity greater in healthy donors than in PVL patients ($p < 3.688e\text{-}05$).

Microbial alpha and beta diversity were evaluated using richness (Chao1, Alpha1), evenness (Shannon-Wiener), dominance (Simpson, Inverse Simpson), and similarity (Jaccard and Sorensen) metrics (Fig. 2). Both indices used to assess richness were significantly different between PVL and healthy samples (Fig. 2A and 2B). Certainly, the average number of observed OTUs found was higher for healthy donors than for PVL (251 vs 282, $p < 0.05$). No significant differences were found in terms of evenness (Fig. 2C) or dominance (Fig. 2D and 2E). Regarding similarity, three out of five samples from healthy donors showed significant differences with the rest of samples (Fig. 2F and 2G). The oral sample from PVL_1 patient also showed differences with samples from PVL_3, PVL_7, and PVL_10 patients. A prominent feature of oral microbiome evidenced from these results is that there is a high degree of inter-individual variability in community composition among study participants especially in the healthy subgroup.

Krona plots representing an overview of the oral microbiome of healthy controls and PVL patients are available in Supplementary Material S1 and S2, respectively. The most abundant phyla found in PVL included *Firmicutes* (45%), *Fusobacteria* (17%), *Proteobacteria* (14%), *Actinobacteria* (12%), and *Bacteroidetes* (9%), whereas in healthy controls included *Firmicutes* (51%), *Fusobacteria* (13%), *Proteobacteria* (11%), *Actinobacteria* (9%), *Bacteroidetes* (8%), *Spirochaetes* (2%), and *Planctomycetes* (2%).

At the genus level, the most frequently detected genera in PVL were *Streptococcus* (28%), *Gemella* (4%), *Veillonella* (2%), and *Granulicatella* (2%) of *Firmicutes; Fusobacterium* (13%), and *Leptotrichia* (5%) of *Fusobacteria*; *Haemophilus* (7%), *Campylobacter* (3%), *Aggregatibacter* (2%), and *Neisseria* (2%) of *Proteobacteria*; *Rothia* (8%) and *Corynebacterium* (2%) of *Actinobacteria*; and *Prevotella* (5%) and *Porphyromonas* (2%) of *Bacteroidetes*. Regarding healthy controls, *Streptococcus* (22%), *Gemella* (4%), *Granulicatella* (3%), *Bacillus* (2%), *Exiguobacterium* (2%), *Filifactor* (1%), and *Dialister* (1%) of *Firmicutes; Fusobacterium* (11%), and *Leptotrichia* (2%) of *Fusobacteria*; *Haemophilus* (6%), and *Candidatus Carsonella* (1%) of *Proteobacteria*; *Rothia* (5%) and *Corynebacterium* (1%) of *Actinobacteria*; *Prevotella* (2%), *Porphyromonas* (2%), and *Alloprevotella* (1%) of *Bacteroidetes*, *Treponema* (2%) of *Spirochaetes*; *Gemmata* (2%) of *Planctomycetes;* and *TM7* (1%) were the most abundant.

Community structure reveals differently abundant OTUs in PVL and healthy oral samples

In addition to the differences found in microbiome composition in terms of overall diversity, specific OTUs were identified that exhibited differences in abundance between PVL and healthy samples. Prior to further analysis, OTUs were filtered in order to remove microorganisms with very low counts across all libraries since they provide little evidence for differential distribution analysis. After filtering, 110 genera were found to be exclusively present in healthy donors, whereas 14 were exclusive of PVL (Fig. 3A). The differential distribution analysis revealed that oral samples from healthy donors were significantly enriched in

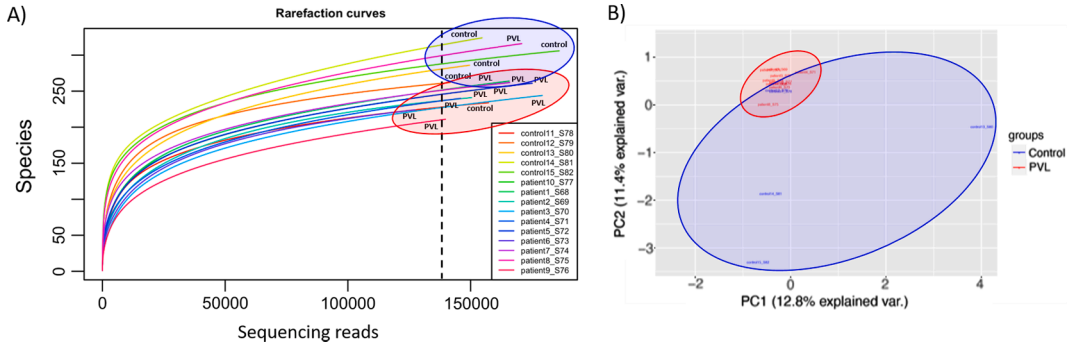

Fig. 1. Microbiome diversity in samples from PVL and healthy controls. (A) Rarefaction curves. Average number of OTUs detected versus sequencing library size for oral samples collected from PVL patients and healthy controls. (B) PCA score plot of samples according to their microbiome.

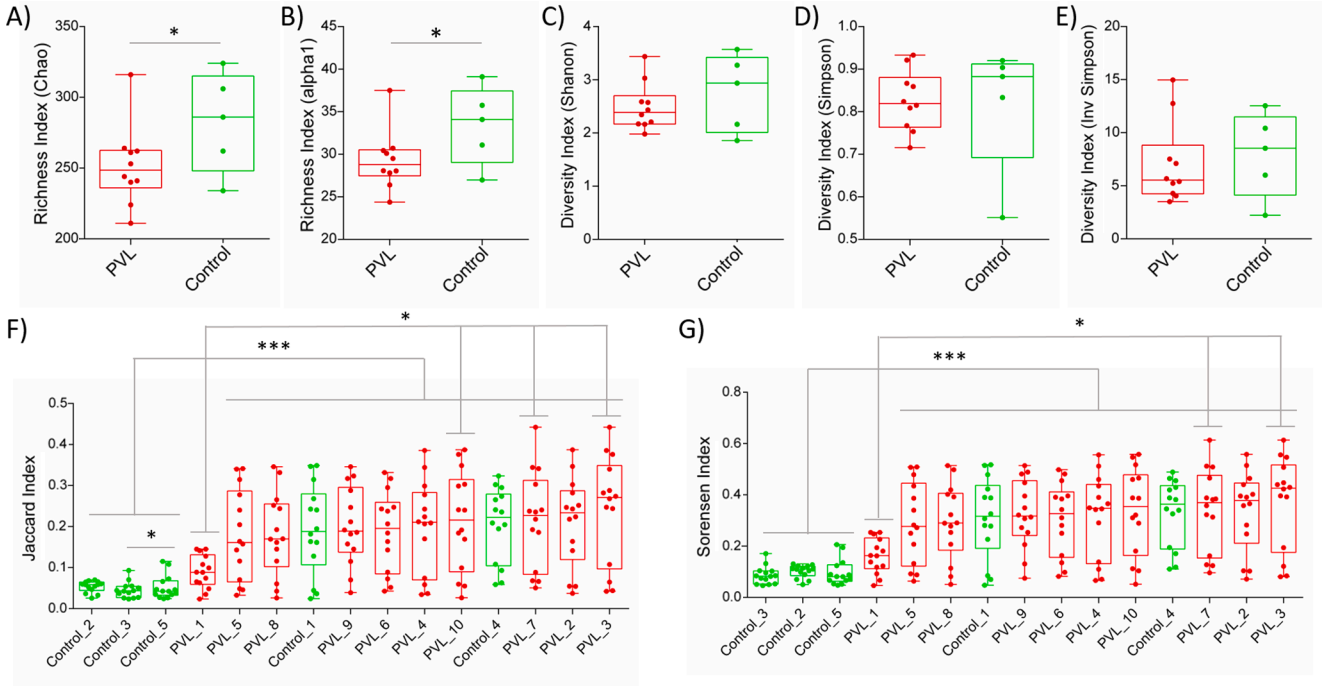

Fig. 2. Microbial richness, evenness, dominance, and similarity of oral samples. (A) Chao, (B) Alpha1, (C) Shannon, (D) Simpson, (E) Inverse Simpson, (F) Jaccard, (G) Sorensen indexes. *p < 0.05, ***p < 0.001.

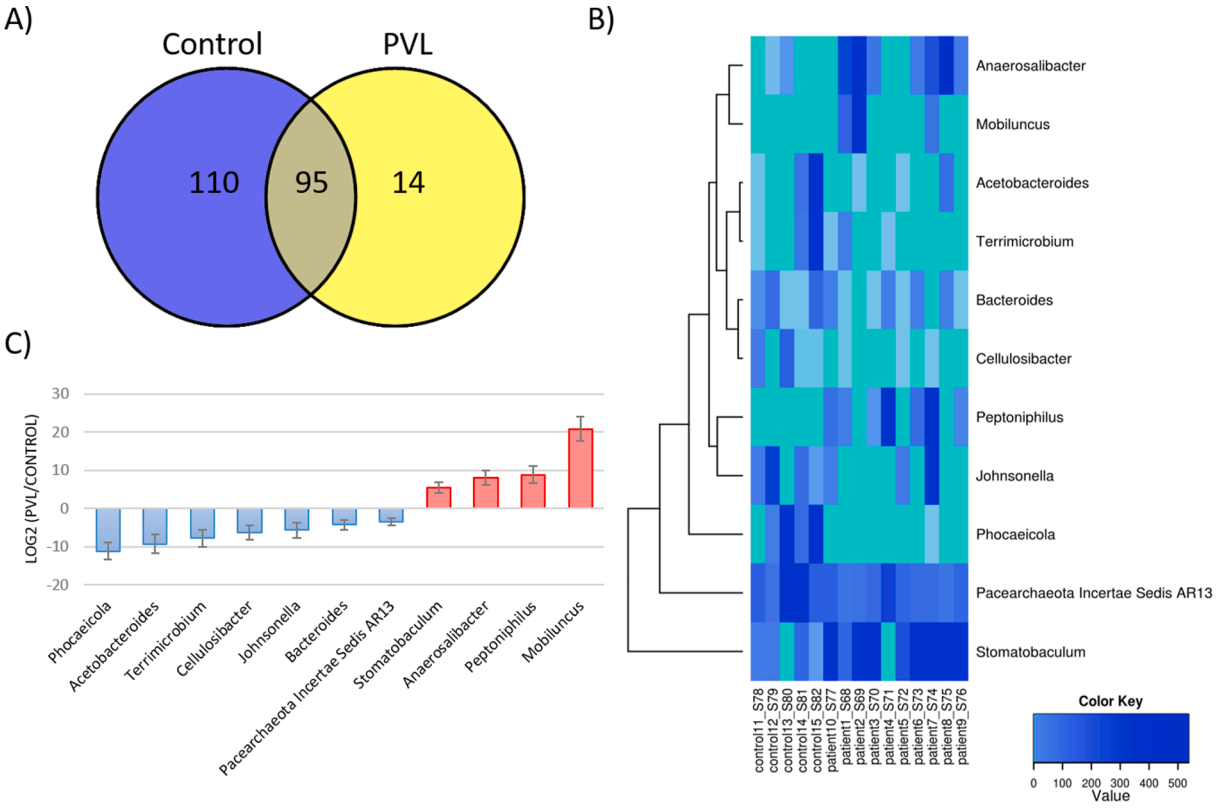

Fig. 3. Differently abundant genera between oral samples from PVL patients and healthy controls. (A) Venn diagram summarizing the distribution of genera along groups. (B) Clustering of the differentially distributed genera along samples. (C) Log2 fold change of the abundances of genera showing significant difference between PVL and control at a false discovery rate of 5%.

Pacearchaeota Incertae Sedis AR13, *Bacteroides*, *Johnsonella*, *Cellulosibacter*, *Terrimicrobium*, *Acetobacteroides*, and *Phocaeicola*, whereas PVL patients were enriched in *Mobiluncus*, *Peptoniphilus*, *Anaerosalibacter*, and *Stomatobaculum* genera (Fig. 3B and 3C).

At species level, 171 OTUs were found to be exclusive of healthy samples, whereas 25 were exclusively present in PVL (Fig. 4A). In this

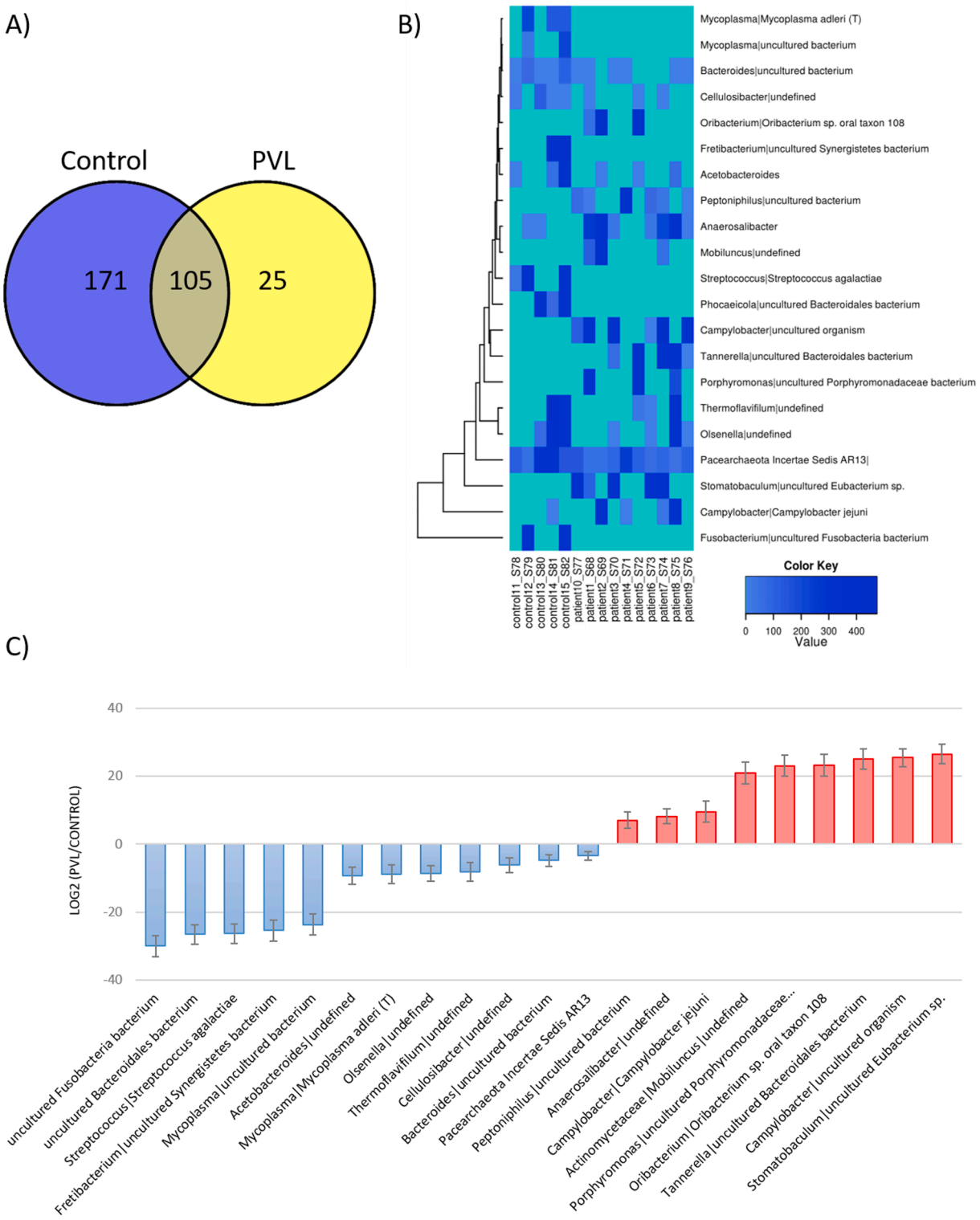

Fig. 4. Differently abundant species between oral samples from PVL patients and healthy controls. (A) Venn diagram summarizing the distribution of species along groups. (B) Clustering of the differentially distributed species along samples. (C) Log2 fold change of the abundances of species showing significant difference between PVL and control at a false discovery rate of 5%.

case, the differential distribution analysis revealed that oral samples from PVL were significantly enriched in *Oribacterium* sp. *oral taxon 108*, *Campylobacter jejuni*, uncultured *Eubacterium* sp., *Campylobacter*, *Tannerella*, *Porphyromonas*, *Peptoniphilus*, and undefined *Mobiluncus* and *Anaerosalibacter* bacteria (Fig. 4B and 4C). On the other hand, healthy samples were significantly enriched in *Streptococcus agalactiae*, *Mycoplasma adleri (T)*, uncultured *Fusobacteria*, *Bacteroidales*, *Synergistetes*, *Mycoplasma*, *Bacteroides*, and undefined *Acetobacteroides*, *Olsenella*, *Thermoflavifilum*, *Cellulosibacter*, and *Pacearchaeota Incertae Sedis AR13*.

Discussion

Human oral cavity harbors the second most abundant microbiota after the gastrointestinal tract. As of March 2021, the expanded Human

Oral Microbiome Database (eHOMD) contains information of 775 prokaryotic species, where 70% are cultivable, and 30% belong to the uncultivable class of microorganisms. These hidden oral micro-inhabitants exhibit a direct influence on human health, from host's metabolism to immune responses. Altered oral microbiota has been observed in a wide range of diseases, including diabetes, bacteremia, endocarditis, cancer, autoimmune disease and atherosclerosis [45–49]. Therefore, it becomes crucial to understand the oral microbial diversity and how it fluctuates under diseased/perturbed conditions.

In this study, we characterized oral microbial communities of PVL patients and compared it with those of disease free, age-matched controls. In terms of overall diversity, the average number of observed OTUs found was significantly higher for healthy donors than for PVL. In concordance with our results, reduction in oral microbial diversity have been consistently reported in other oral diseases such as caries, recurrent aphthous stomatitis, oral lichen planus, and nasopharyngeal carcinoma [50–52]. We also found a high inter-individual variability, observed between microbiomes of the participants in our study. These variations have been repeatedly reported in publications on oral microbiome [52–54], which makes it challenging to define what constitutes the 'core' oral microbiome at low taxonomic ranks. At phylum level, *Firmicutes* (36.7%), *Bacteroidetes* (17.1%), *Proteobacteria* (17.1%), *Actinobacteria* (11.6%), *Spirochaetes* (7.9%) and *Fusobacteria* (5.2%) have been reported to constitute the 96% of the total oral bacteria [55] which is in consonance with the most abundant phyla found for both groups in our study.

The investigation carried out by Bik et al. 2010 further extends core microbiome at the genus level. Accordingly, the major constituents of the core microbiome of oral cavity include *Granulicatella*, *Streptococcus*, and *Veillonella* of *Firmicutes*; *Campylobacter*, *Cardiobacterium*, *Haemophilus*, *Neisseria* of *Proteobacteria*; *Actinomyces*, *Atopobium*, *Corynebacterium*, *Rothia of Actinobacteria*; *Bergeyella*, *Capnocytophaga*, *Prevotella* of *Bacteroidetes*; TM7, and *Fusobacterium* [56]. However, differences can be found between studies, since many factors, including smoking habits, diet, and varying geographical and climatic conditions significantly alter the oral microbiota, making difficult the comparisons [57]. In our study, these bacteria were the most frequently detected genera in all the patients included, except for *Cardiobacterium*, *Actinomyces*, *Atopobium, Bergeyella* and *Capnocytophaga*, which were less frequently detected than *Gemella*, *Leptotrichia*, or *Porphyromonas*. In addition, archaea were also detected, which has been previously reported in the oral cavity [58,59]. The presence of *Bacillus*, *Exiguobacterium*, *Filifactor*, *Dialister*, *Candidatus Carsonella*, *Alloprevotella*, *Treponema*, and *Gemmata* was also remarkable in healthy donors, whereas PVL patients were enriched in *Aggregatibacter*. This genus is often found in association with localized aggressive periodontitis and has been seen to modulate the host immune response through CDT, leukotoxin, and LPS [60]. In consonance, the pathophysiologic inflammatory responses associated to *Aggregatibacter* in periodontal disease could also be of relevance for PVL. In addition to the differences exposed in overall microbiome profiles, we also identified several OTUs that were significantly over-represented in PVL or healthy subgroups. Of note, *Oribacterium* sp. *oral taxon 108* and *Campylobacter jejuni* were found more abundant in PVL patients. *Oribacterium* sp. *oral taxon 108* is a strictly anaerobic microbe that was recently found to be more abundant in saliva samples from acute leukemia patients than in healthy controls [61]. On its regard, *Campylobacter jejuni* has been shown to promote intestinal inflammation and colorectal tumorigenesis through the action of CDT, which has DNA damage potential [62]. Moreover, uncultured *Eubacterium*, *Tannerella* and *Porphyromonas* were also more abundant in PVL patients than in healthy donors. Unculturable *Eubacterium* has been reported as a putative periodontal pathogen [54] whereas *Tannerella* and *Porphyromonas* have been associated to periodontitis and head and neck cancers [63–65]. On the contrary, several archaea and bacteria were found to be enriched in healthy donors. Interactions between different microbial species are increasingly recognized as important determinants of microbial colonization patterns since these interactions could determine the composition of the oral microbiome, acting with probiotic potential [66]. For instance, *Streptococcus salivarius* has been reported to produce inhibitory molecules that provide antimicrobial activity against *Streptococcus pneumoniae* [67].

To date, numerous articles have focused on OSCC microbiome, but the literature is sparse about the microbiome associated to oral leukoplakia [68]. Amer and colleagues compared swabs from oral leukoplakia to contralateral healthy site and controls [69]. In consonance with our results, healthy controls exhibited greater richness than oral leukoplakia patients. However, oral leukoplakia patients reveal differences between the oral leukoplakia and the healthy donors, with *Rothia mucilaginosa, Alloprevotella* sp., *Neisseria meningitides,* and *Leptotrichia* sp. significantly elevated and *Neisseria oralis, Streptococcus infantis,* and *Lautropia mirabilis* significantly diminished in oral leukoplakia compared to control [69]. Another study conducted in saliva samples concluded that the microbiome of oral leukoplakia patients had *Haemophilus* significantly elevated and *Bacillus* and *Abitrophia* significantly decreased compared to controls [70]. The diverse results reported might seem discouraging, but as aforementioned, many factors, including sampling, smoking habits, alcohol consumption, diet or geographical and climatic conditions hamper a reliable comparison. For instance, smoking has been reported to have more significant impact on community structure than whether the sample was recovered from oral leukoplakia [69]. Although smoking is less frequent in PVL patients than in other oral leukoplakias, a 30% of the PVL patients included in this study were smokers with severe or moderate dysplasia who developed OSCC during the follow-up.

To the best of our knowledge, this is the first study investigating the oral microbiome alterations in PVL. As with other studies comparing disease versus healthy microbiome, it is not possible to say whether the microbial alterations found are the cause or the consequence of the disease. In addition, it shows limitations regarding the limited sample size and the tracking of factors like the oral hygiene and periodontal status. As a result, further longitudinal studies with larger sample size and animal model studies are needed to clarify the role of microbiome in PVL development. Given that the diagnosis of PVL is an issue of debate in numerous scientific papers, the identification of oral microbiota-based biomarkers can be extremely helpful, at least when talking about the aetiology of this unknown disorder. Additionally, further studies could also determine if oral microbiome have a role stratifying PVL patient outcomes.

Conclusion

Using a 16S rRNA gene sequencing-based approach, oral microbial dysbiosis was found to be a common state in patients with PVL. Loss of diversity and enrichment of pathogens such as *Oribacterium* sp. *oral taxon 108*, *Campylobacter jejuni*, *Eubacterium* sp., *Tannerella*, or *Porphyromonas* may be important risks for PVL development. A better understanding of the role of oral microbiome in oral leukoplakias could direct to novel non-invasive diagnostic and prognostic options, as well as to more personalized treatments and microbiome-targeted therapeutic interventions.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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