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ORIGINAL PAPER

The role of oral microbiome in pemphigus vulgaris

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Abstract

While the impact of oral microbiome dysbiosis on autoimmune diseases has been partially investigated, its role on bullous diseases like Pemphigus Vulgaris (PV) is a totally unexplored field. This study aims to present the composition and relative abundance of microbial communities in both healthy individuals and patients with oral PV lesions. Ion Torrent was used to apply deep sequencing of the bacterial 16S rRNA gene to oral smear samples of 15 healthy subjects and 15 patients. The results showed that the most dominant phyla were *Firmicutes* (55.88% controls-c vs 61.27% patients-p, p value = 0.002), *Proteobacteria* (9.17%c vs 12.33%p, p value = 0.007) and *Fusobacteria* (3.39%c vs 4.09%p, p value = 0.03). Alpha diversity showed a significant difference in the number of genera between patients and controls (p value = 0.04). Beta diversity showed statistical differences in the microbial community composition between two groups. *Fusobacterium nucleatum*, *Gemella haemolysans* and *Parvimonas micra* were statistically abundant in patients. We noticed the characteristic fetor coming out of oral PV lesions. Most of anaerobic bacteria responsible for oral halitosis are periopathogenic. Though, only *F. nucleatum* and *P. micra* were differentially abundant in our patients. Especially, *F. nucleatum* has been reported many times as responsible for bad breath. Furthermore, *Streptococcus salivarius* and *Rothia mucilaginosa*, species mostly associated with clean breath, were found in relative abundance in the healthy group. Consequently, the distinct malodor observed in PV patients might be attributed either to the abundance of *F. nucleatum* and *P. micra* and/or to the lower levels of *S. salivarius* and *R. mucilanginosa* in oral lesions

Keywords Oral microbiome · Dysbiosis · Next-generation sequence · Pemphigus vulgaris · Halitosis

Introduction

Pemphigus is a group of rare, chronic, potentially life-threatening autoimmune mucocutaneous disease, characterized by epithelial blistering affecting cutaneous and/or mucosal surfaces, the term derived from the Greek word 'Pemphix' meaning blister. Pemphigus vulgaris (PV) is the most common type of pemphigus accounting for approximately 70% of pemphigus cases. It is characterized by suprabasal acantholysis (Hashimoto 2003).

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With regard to PV etiology, there may be a complex interaction between genetic factors and environmental triggering factors such as drug intake, diet, physical agents (UV radiation, ionizing radiation, thermal and electrical burns, surgery and cosmetic procedures), emotional stress, infections (primarily viral infections), contact allergens, pregnancy, etc. that may contribute to the disease cause or manifestation (Ruocco et al. 2013).

In more than 50% of cases, the oral mucosa is the site of onset of the disease's manifestations. Oral lesions may precede cutaneous lesions by several months or may remain the only manifestations in some patients (Kuriachan et al. 2015).

The oral cavity offers an ideal environment for the colonization and growth of microorganisms as it provides warmth, humidity and nutritional abundance. The oral microbiome (OMB) is the second most complex one in the human body after that of the intestine. Almost 1000 different species of bacteria can be detected in the oral cavity (Paster et al. 2000). Saliva, oral mucosa and tooth surfaces are the three available sites in the human mouth for microbial colonization leading to the formation of respective microbiomes.

OMB can be studied by several techniques. Currently, the most effective is next-generation sequencing (NGS) of microorganisms' genomes which constitutes a breakthrough in DNA sequencing. It offers parallel, massive, deep sequencing of entire genomes at unprecedented speed and low cost (Vincent et al. 2017).

The most frequently used gene is the one responsible for the 16S rRNA coding (Pozhitkov et al. 2005).

Plenty of studies have investigated the relationship between the OMB and systemic and distant diseases, including cardiovascular diseases, tumor, cerebral or hepatic abscesses, cystic fibrosis, diabetes mellitus, obesity, miscarriage or low weight infant (Mitchell-Lewis et al. 2001; Schiff et al. 2003; Marques da Silva et al. 2004; Rogers et al. 2006; Koren et al. 2011; Farrell et al. 2012; Kumar et al. 2014; Mervish et al. 2019).

Furthermore, accumulating research evidence suggests that the dramatic increase in the incidence of several autoimmune and inflammatory diseases noticed in the developed world during the last decade might be the result of host–microorganism equilibrium disturbance (dysbiosis) (Levy et al. 2017; De Luca and Shoenfeld 2019). Specifically, the OMB has been linked to the pathogenesis of some autoimmune diseases such as Sjögren's syndrome, systemic lupus erythematosus, rheumatoid arthritis, Behcet's disease, Crohn's disease and psoriasis (Zorba et al. 2020).

Concerning autoimmune bullous diseases, while a remarkable progress has been achieved in the identification of genetic factors and the development, progress and severity of these diseases, no environmental agent has been conclusively identified. Studies on the effect of the microbiome on disease inducement and manifestation are few and far between (Ellebrecht et al. 2016; Miodovnik et al. 2017).

The exact mechanism responsible for the induction of autoimmunity by the action of infectious agents remains unknown. Experiments on animal models have shown that activation of autoreactive T cells is a cornerstone for the development of an autoimmune process (Wucherpfennig 2001). Several mechanisms have been proposed either separately or in various combinations to explain the association of autoimmunity and infection. Molecular mimicry, bystander activation and stimulation of Pattern Recognition Receptors (PRR), epitope spreading, viral and bacterial superantigens and viral persistence with polyclonal activation of B cells are the most frequently mentioned (Chervonsky 2013; Imenez-Dalmaroni et al. 2016). The aim of the present study is to characterize the relative abundance of microbial communities of the oral cavity at the phylum, family, genus and species levels, by deep sequencing of the bacterial 16S rRNA gene in both healthy individuals and patients with PV. The results of this study will contribute to further investigation of the relationship between OMB and PV.

Materials and methods

This study was designed according to the principles of Helsinki's Declaration and was approved by the Committee on Ethics of Aristotle University of Thessaloniki.

The study comprises the test group and the control group. The first one includes 15 PV patients (9 female and 6 male) from the Autoimmune Bullous Diseases Outpatient Clinic of the 2nd Dermatology Department of Medicine School of Aristotle University of Thessaloniki which were recruited between January 2016 and December 2018. The latter one includes 15 healthy controls with no oral and cutaneous clinical signs of bullous disease which were recruited from the Oral Medicine and Oral Pathology Clinic of Dental School of Aristotle University of Thessaloniki.

Regarding the test group, inclusion criteria consisted of confirmed diagnosis of PV which was based on clinical, histopathological and immunological investigations. The laboratory tests used were biopsy, direct immunofluorescence (DIF), indirect immunofluorescence (IIF) (with monkey esophagus substrate) and detection of serum circulating autoantibodies against desmoglein-1 and desmoglein-3 (anti-Dsg1 and anti-Dsg2) with enzyme-linked immunosorbent assay (ELISA). The definitive diagnosis was set when biopsy and at least one of the DIF, IIF or serology test (anti-Dsg antibodies) were consistent with PV.

Exclusion criteria included:

1. Apparent clinical signs of intraoral fungal or viral infection
2. Therapy for the last 6 months with:

   (i) Systemic (per os, intravenous or intramuscular) antimicrobial chemotherapeutic, antifungal, antiviral or other antiparasitic drugs.
   (ii) Steroid drugs (per os, intravenous, intramuscular or inhaled).
   (iii) Immunomodulators
   (iv) Immunosuppressive drugs (such as methotrexate, cyclosporine, antineoplasmatic drugs).
   (v) Commercial probiotics in doses greater than or equal to 10^8 cfu (colony-forming units)/24 h.
   (vi) Therapy for the last 7 days with topical corticosteroids (cream, mouthwash).
   (vii) Therapy for the last 48 h with antimicrobial or antiseptic drugs in the form of mouthwash.

Written detailed consent forms and questionnaire data sheets were obtained from both groups who agreed to serve as oral smear donors. Healthy control individuals were matched for age, sex and smoking to the patients. All subjects were of Caucasian race with an age range between 37 and 83 years. There were nine females who prevailed over six males resulting in a proportion of 3:2. Descriptive outlines for all subjects are presented in Supplemental File 1.

Each smear sample from the control group was obtained from healthy areas of oral mucosa while the respective sample from the test group was obtained from oral lesions of PV. From both groups, mucosal sites in contact with teeth as well as gingival sites were excluded from the sampling procedure to avoid confusion with the microorganisms of dental plaque.

Sample collection and storage

The sampling procedure commenced immediately before the beginning of systemic corticosteroid therapy for the disease. Using special sterile cotton swabs (Sterile Catch-All™ Specimen Collection Swabs/Epicentre Biotechnologies, Madison WI), each sample was collected after gentle rotary motions on the intraoral mucosal PV lesions for 10 s. Isolation of lesions from the rest of the oral mucosa and dental tissues by means of cotton tulip and air syringes were preceded. After collecting the smear, the swab was inserted into a 2 ml tube with a screw cap containing 750 µl lysis buffer. The swab was pressed against the walls of the tube for 20 s to ensure the transfer of the microorganisms to the lysis fluid. The tubes were then transported by means of a special iced bag to the 2nd Laboratory of Microbiology of Medical School of Aristotle University of Thessaloniki, where the samples were stored in a freezer at − 80° C until further processing.

DNA extraction

For each subject, total genomic DNA extracted from 200 µl of sample according to the manufacturer's recommended procedure using the Qiagen DNA mini kit (Qiagen, Hilden, Germany). The DNA was eluted in 100 µl of AE elution buffer (Qiagen, Hilden, Germany) and stored at − 20 °C until use. Total DNA concentration of each DNA extract was measured using Qubit fluorometer (Life Technologies Corporation, Carlsbad, CA, USA) with the Qubit dsDNA HS assay (Life Technologies Corporation, Carlsbad, CA, USA).

16S rRNA amplicon preparation, sequencing, and analysis

Library preparation

DNA libraries for next-generation sequencing were constructed using two primer sets that selectively amplify the corresponding hypervariable regions of the 16 s region in bacteria (V2, 3, 4, 6, 7, 8, 9). The primers used for amplification contain adaptors for Ion Torrent sequencing and Ion Xpress barcodes so that the PCR products could be pooled and sequenced directly. For the library preparation, the end repair was followed by purification of pooled amplicons with AMPure beads, ligation of Ion Xpress barcode adapters and nick-repair, using the Ion Plus Fragment Library kit (Life Technologies Corporation, Carlsbad, CA, USA). Purification steps were performed using AMPure beads (Agencourt), eluted in low Tris–EDTA (TE) buffer, and quantified by using a Qubit dsDNA HS (high sensitivity) kit (Life Technologies Corporation, Carlsbad, CA, USA). The library concentration was determined using Ion Universal Library Quantification kit (Life Technologies Corporation, Carlsbad, CA, USA) in an ABI7500 real time PCR system. The two resulting PCR libraries were equimolarly pooled after DNA purification.

Template preparation and sequencing

Before emulsion PCR, the library concentration was adjusted to 26 pM. Template preparation was performed in Ion 400 Template One-Touch 2 following enrichment of the amplified Ion Sphere Particles using Dynalbeads MyOne Streptavidin C1 beads (Life Technologies Corporation, Carlsbad, CA, USA) using the Ion One-Touch ES system according to the manufacturer's protocol.

The Ion Xpress barcoded library was sequenced using the Personal Genome Machine (PGM), Ion Torrent (Life Technologies Corporation, Carlsbad, CA, USA). A total of 10 barcoded samples were pooled and loaded into each 316 chip (Life Technologies, MA, USA) using the Ion PGM 400

Sequencing Kit (Life Technologies Corporation, Carlsbad, CA, USA), according to the manufacturer's instructions.

16 s rRNA sequence data preprocessing

Base calling and run demultiplexing were performed using the Torrent Server software. The sequences were analyzed using the metagenomics workflow in the Ion Reporter™ Software that enables the identification, at the genus or species level, of microbes present in complex multi-bacterial samples, and uses both the premium curated MicroSEQ™ ID 16S rRNA reference database and the curated Greengenes database.

Sequencing of the 30 clinical samples on the Ion Torrent PGM instrument generated 570.684 total raw reads. Raw reads were processed using the Ion Reporter software, which performs demultiplexing and denoising, quality filtering, alignment against a reference database of 16SrRNA gene sequences and clustering into operational taxonomic units (OTUs) at 97% similarity. OTUs were assigned taxonomy using the Greengenes database and analysis was performed using Quantitative Insights into Microbial Ecology (QIIME).

16S rRNA sequencing data analysis

The open source R programming language 3.5.2v was used for microbiome data processing (R Development Core Team). Specifically, vegan 2.5.3v, phyloseq 1.24.2v and ggplot2 3.1.0v R packages were imported for data analysis and data visualization (Oksanen et al. 2018; Wickham 2009; McMurdie and Holmes 2013). The relative abundance of taxa at phylum, family, genus and species level was explored with the Kruskal–Wallis non-parametric test, bar plots, heatmap plots and Venn diagrams.

Microbial diversity within each sample was evaluated through Shannon, Simpson and Fisher a-diversity indexes. Differences in a-diversity among patients and controls were detected with the Kruskal–Wallis non-parametric test. Differentially abundant taxa were detected with Linear discriminant analysis Effect Size (LEfSe) method, with threshold LDA value 2.0 (Segata et al. 2011). LEfSe analysis was implemented for patients vs control subjects, and in subgroups of patients, categorized in accordance with smoking.

Regarding b-diversity between samples, Bray–Curtis dissimilarity matrix was calculated and Principal Coordinate Analysis (PCoA) was applied for finding out taxa that were count more in the total variance and for pattern visualization. Permutational Multivariate Analysis of Variance (PERMANOVA) with 1000 permutations was implemented together with PERMutational analysis of multivariate DISPersion (PERMDISP) to confirm significant differences between groups (Anderson 2006, 2008; Anderson et al. 2006).

Results

Exploration of relative abundance

In total 8 phyla and 32 families were identified in controls and 9 phyla and 51 families in patients. Mean number of raw reads at family level was much higher in patients that in controls (Supplemental file 2).

The summarized results concerning relative abundance in control group and in patients in phylum and family level as well as those depicting statistical difference between the two groups are provided in Table 1.

At phylum level nine phyla and 52 families were identified in all of the 30 samples in patients and control subjects. The most abundant phyla detected were *Firmicutes* (60.0%) and *Bacteroidetes* (16.20%), followed by *Proteobacteria* (11.59%), *Actinobacteria* (7.24%) and *Fusobacteria* (3.94%). The remaining four phyla *Cyanobacteria*, *Chloroflexi*, *Spirochaetes* and *Tenericutes* contributed lower than 2.0% to the total abundance of bacteria in the OMB. Phylum *Chloroflexi* was detected only in three patients contributing 0.11% of total abundance of bacteria in the OMB. Percentages are expressed in percent of total reads.

At genus level, from the ten most abundant genera detected, *Streptococcus* (33.30%c vs 34.37%p, p value = 0.006), *Fusobacterium* (4.13%c-controls vs 4.51%p-patients, p value = 0.024) and *Gemella* (5.80%c vs 7.13%p, p value = 0.030) were more abundant in patients with statistical significance.

Table 1 Exploration of relative abundance in phylum and family level

	Healthy controls	patients
Phylum level		
Firmicutes	55.88% (14 families)	**61.27%** (19 families)
Bacteroidetes	18.72% (4)	15.43% (6)
Proteobacteria	9.17% (5)	**12.33%** (3)
Actinobacteria	11.56% (3)	5.91% (16)
Fusobacteria	3.39% (2)	**4.09%** (2)
Family level		
Streptococcaceae	26.28%	29.46%
Pasteurellaceae	16.43%	13.07%
Prevotellaceae	13.17%	10.35%
Porphyromonadaceae	5.34%	8.92%
Neisseriaceae	7.18%	6.43%
Bacillales incertae sedis	1.41%	**5.98%**
Fusobacteriaceae	2.56%	**3.91%**

Highlighted with bold letters are the results showing statistical differential abundance

At species level, 27 species were detected in statistically differentially abundant genera and are shown in Fig. 1.

Exploration of a-diversity in controls versus patients

The alpha diversity was explored at the genus level. The number of genera observed in each subject was higher in patients (p value = 0.04), while no sizable differences were detected from Shannon (p value > 0.05), Simpson (p value = 0.2058) and Fisher indexes (p value = 0.2717) (Supplemental file 3).

Additionally, the LDA Effect Size (LEfSe) method resulted in 41 differentially abundant taxa between patients and control subjects. From these, 11 taxa were significantly increased in abundance in controls and 30 taxa in patients (Fig. 2a).

The results for the control subjects are depicted in Table 2.

The phylum *Firmicutes* and its families *Peptostreptococcaceae* and *Clostridiales FamilyXI Incertae Sedis* were detected with the highest mean in patients. At the genus level, *Selenomonas*, *Actinobacillus* and *Aggregatibacter* were detected. Moreover, genus *Prevotella* with species *maculosa* (1.05% in 5 patients), *nigrescens* (1.30% in 5 patients), *oris* (1.08% in 5 patients) and other *sp* (0.19% in 4 patients) and genus *Streptococcus* with species *intermedius* (0.44% in 5 patients), *mitis* (1.43% in 9 patients), *sanguinis* (0.66% in 4 patients) and *thermophilus* (1.08% in 4 patients) (Fig. 2b).

Furthermore, the analysis resulted in nine significantly discriminative species in patients (Table 3).

Exploration of b-diversity

To explore the beta diversity, OTUs did not appear more than five times in more than 20% of the subjects filtered out. A dissimilarity matrix was calculated at the species level with the Bray–Curtis distance metric for the 40 species that were found after filtering. PCoA was then used for visualization. *X*-axis (PC1) explained 17.5% of the total variance in the data and *y*-axis (PC2) 14.4% (Fig. 3). The PERMANOVA test with 1000 permutations confirmed statistically significant differences between control subjects and patients (p value = 0.0009 < 0.05), which means that there are real differences in the microbial community composition of control subjects and patients. Also, PERMDISP2 procedure lead to insignificant results (p value = 0.05), which means that differences are not due to dispersions of the samples within

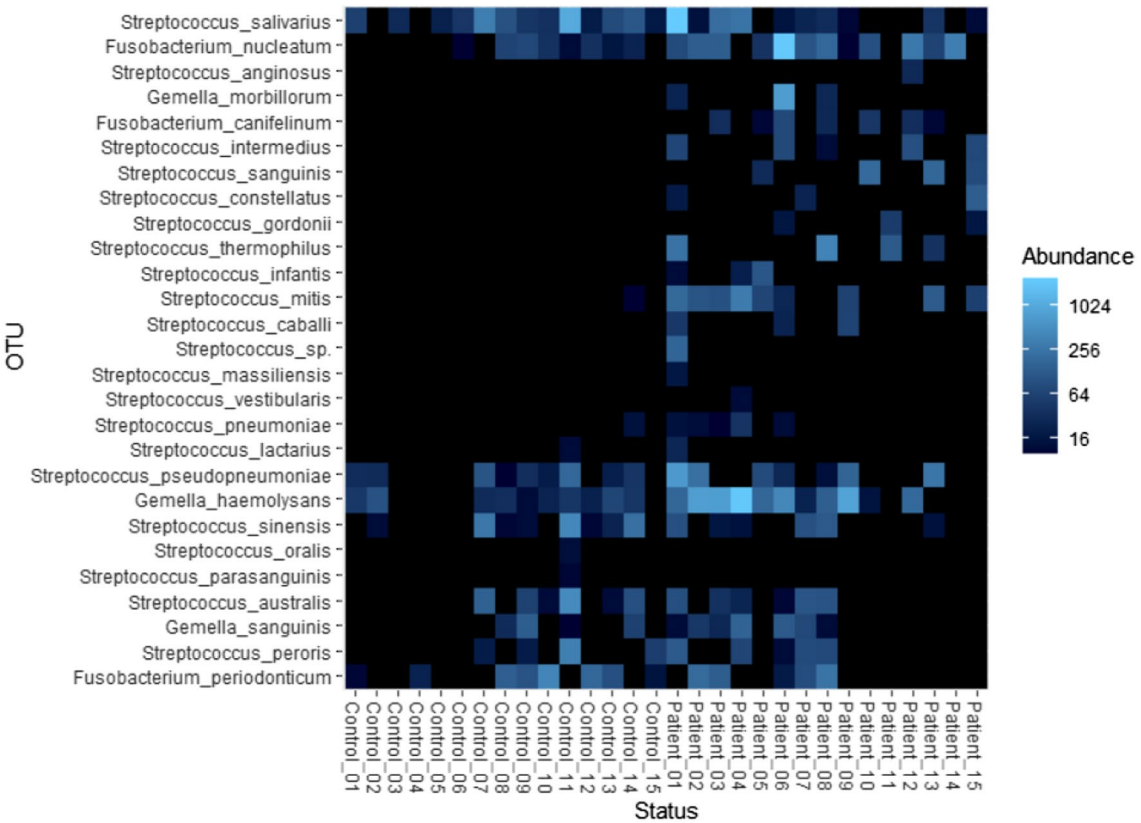

Fig. 1. 27 species detected in statistically differentially abundant genera

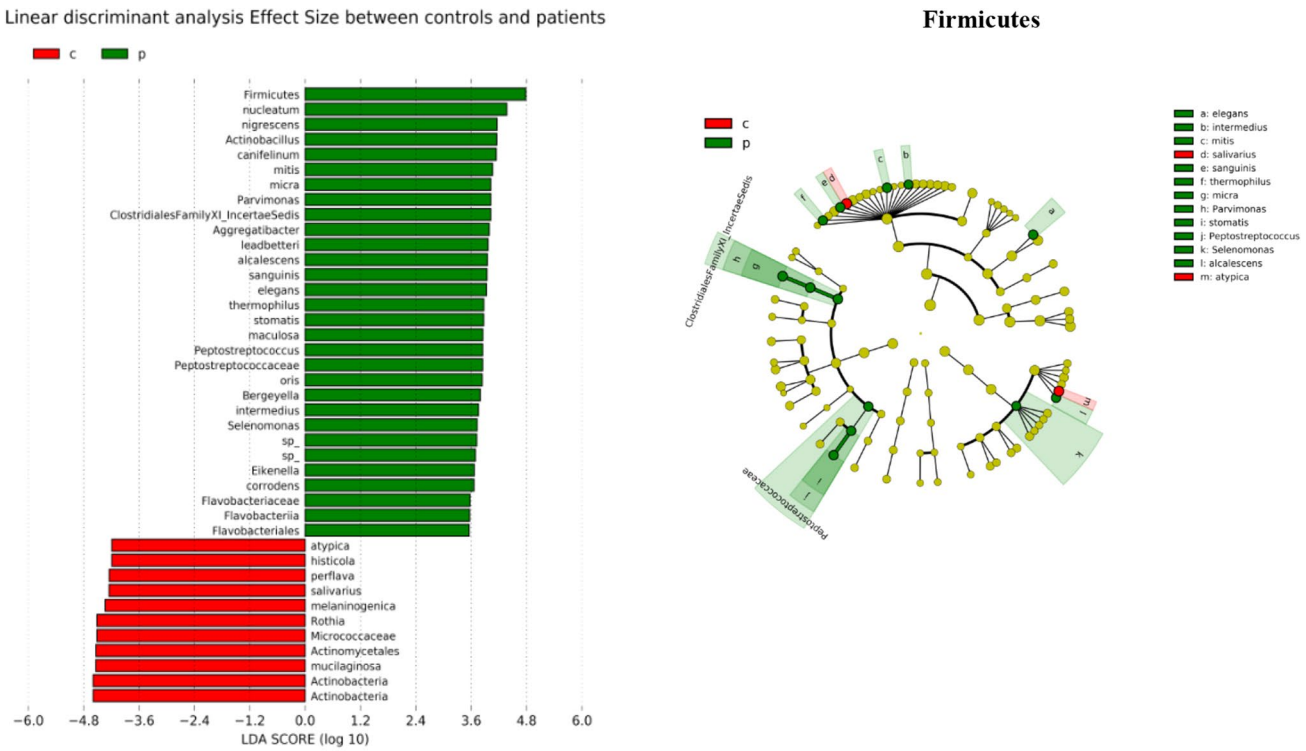

Fig. 2 **a**, **b** LDA Effect Size (LEfSe) method results for patients versus control subjects

Table 2 Species differentially abundant in control subjects

Phylum	Family	Genus	Species	Percentage (%)
Actinobacteria	Micrococcaceae	Rothia	Mucilaginosa	15.22
Bacteroidetes	Prevotellaceae	Prevotella	Histicola	2.47
Bacteroidetes	Prevotellaceae	Prevotella	Melaninogenica	11.26
Firmicutes	Streptococcaceae	Streptococcus	Salivarius	6.26
Firmicutes	Veillonellaceae	Veillonella	Atypica	2.31
Proteobacteria	Neisseriaceae	Neisseria	Perflava	3.54

Table 3 Species differentially abundant in patients

Phylum	Family	Genus	Species	Percentage (%)
Fusobacteria	Fusobacteriaceae	Fusobacterium	nucleatum	5.05
Bacteroidetes	Flavobacteriaceae	Capnocytophaga	leadbetteri	2.60
Firmicutes	ClostridialesFamilyXI_ IncertaeSedis	Parvimonas	micra	1.98
Firmicutes	Carnobacteriaceae	Granulicatella	elegans	1.59
Firmicutes	Peptostreptococcaceae	Peptostreptococcus	stomatis	0.86
Firmicutes	Veillonellaceae	Veillonella	alcalescens	0.83
Fusobacteria	Fusobacteriaceae	Fusobacterium	canifelinum	0.33
Bacteroidetes	Flavobacteriaceae	Bergeyella	sp	0.25
Proteobacteria	Neisseriaceae	Eikenella	corrodens	0.16

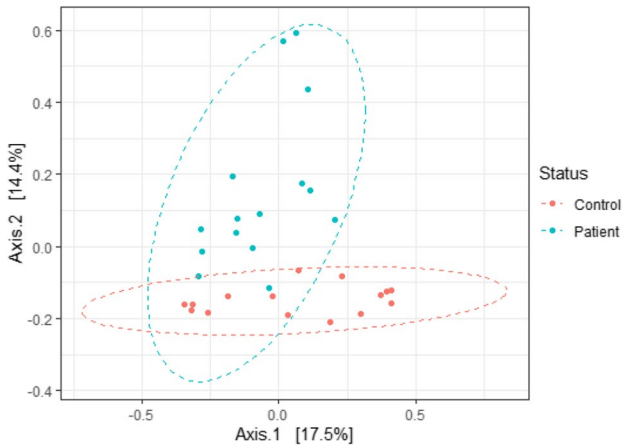

Fig. 3 Exploration of b-diversity

the groups. From the nine species that contributed most to the first two PCs, only *micra* (p value = 0.008) in *Firmicutes* and *nucleatum* (p value = 0.004) in *Fusobacteria* were statistically different in abundance between control subjects and patients.

Exploration based on smoking as an environmental variable

Smokers presented in high abundance the species *Rothia mucilaginosa* (24.29%), *Streptococcus salivarius* (10.26%), *Haemophilus parainfluenzae* (7.95%), *Granulicatella adiacens* (4.24%) and *Streptococcus pseudopneumoniae* (3.81%). They also exhibited high prevalence of *Prevotella melaninogenica* (2.82%) and *Veillonella atypica* (0.49%). Also, the

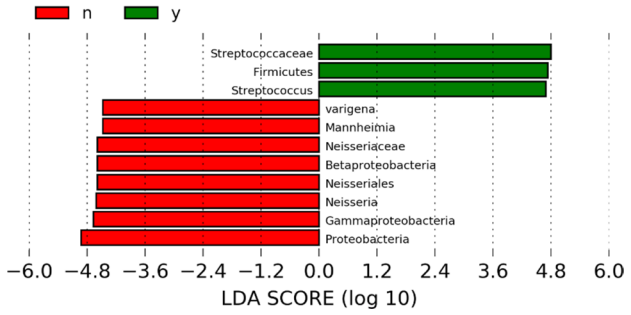

Fig. 5 LEfSe analysis between smokers and non-smokers

Venn diagram (not shown) underlined 11 species only in smokers. These species are presented in the barplot of Fig. 4.

LEfSe analysis between smokers and non-smokers resulted in 11 significantly discriminative taxa. Phylum *Firmicutes* had the highest mean in smokers, while phylum *Proteobacteria* had the highest mean in non-smokers. Details about the remaining taxa, are depicted in the plot in Fig. 5. Also, LEfSe analysis that was performed only to smoking patients resulted in unique species *Neisseria subflava* in phylum *Proteobacteria* and *Prevotella baroniae* in phylum *Bacteroidete* while LEfSe analysis that was performed only to non-smoking patients resulted in unique species *Manheimia varigena.*

Discussion

Up to date, it remains an unexplored field whether OMB could trigger a cascade in the autoimmune response leading to the onset of an autoimmune bullous disease such as

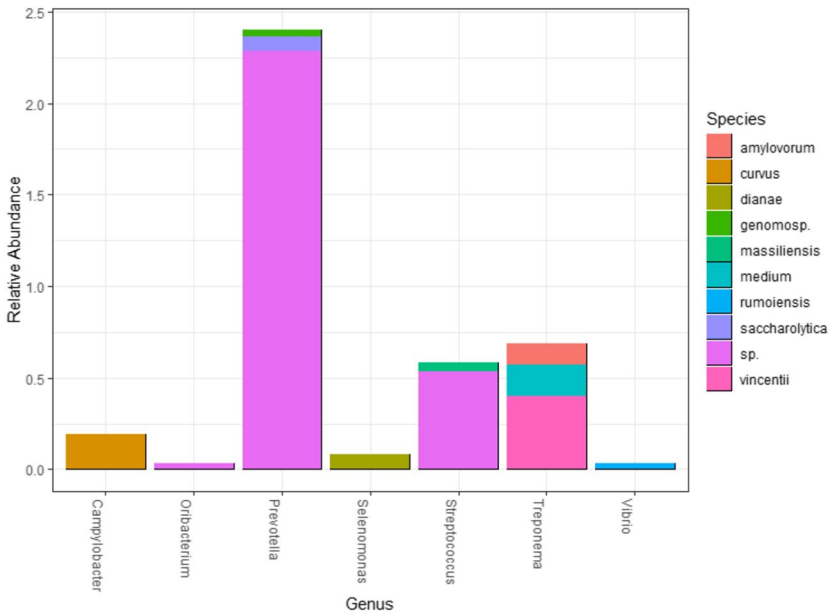

Fig. 4 Genera and species detected only in smokers

pemphigus, mucous membrane pemphigoid, bullous pemphigoid, linear IgA bullous dermatosis, angina bullosa hemorrhagica or epidermolysis bullosa acquisita.

PV is the commonest type of pemphigus group with dominant oral manifestations. There are three published studies that focused on the interaction between cutaneous microbiota and bullous diseases (Ellebrecht et al. 2016; Miodovnik et al. 2017; Scaglione et al. 2020). But so far, only one of these investigations evaluated OMB in patients with oral PV (Scaglione et al. 2020).

Particularly, Ellebrecht et al. pointed out that regardless of the presence of circulating autoantibodies in patients with epidermolysis bullosa acquisita, what plays a significant role in the manifestation of the disease is the composition of the skin microbiome and that the dominance of certain bacterial species appears to be protective against disease manifestation (Ellebrecht et al. 2016). Moreover, Miodovnik et al. reported the existence of a distinct cutaneous microbiota profile in bullous pemphigoid patients (Miodovnik et al. 2017).

In this present study, we used NGS technology to evaluate in depth the microbial composition of oral lesions in PV in an effort to shed light on the interaction between OMB and PV. The findings of this study have to be seen in light of some limitations. The first is the lack of previous investigations, except one that limits the ability to compare our results with other similar studies. The second, major limitation concerns the small sample size that is due to the rarity of the disease and the strict exclusion criteria. It is, though, larger than that collected by the other available study which is mentioned below.

At the phylum level, we detected nine phyla in total samples. The most abundant phyla were *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria* and *Fusobacteria* and were the same in healthy subjects and patients with some small difference in the relative abundance of *Actinobacteria* vs *Proteobacteria*. Additionally, we found a statistically significant dominance of *Firmicutes*, *Proteobacteria* and *Fusobacteria* in patients compared to healthy controls.

These findings are in accordance with those referred to the literature about OMB in healthy individuals (Lazarevic et al. 2010; Ahn et al. 2011; Stahringer et al. 2012; Huttenhower et al. 2012). In patients, our observations share some similarities with those published by Scaglione et al. in a study which is the only one available that provides information about microbial composition in oral PV lesions (Scaglione et al. 2020). The researchers remarked that *Firmicutes* phylum was at higher relative abundance followed by *Fusobacteria*, *Proteobacteria*, *Actinobacteria* and *Bacteroidetes*. Specifically, *Bacteroidetes* phylum was significantly underrepresented compared to healthy controls. The authors assumed that there may be an association between the latter observation and the typical PV fetor. Lastly, *Staphylococcus* genus was the most represented in PV oral samples, while in our findings, *Streptococcus* was the statistically dominant genus in patients.

Exploring the alpha diversity at the genus level, the number of genera observed in each subject was higher in patients than healthy controls. This microbial richness in patients might be correlated to the inadequate application of oral hygiene rules due to painful oral lesions.

Analyzing the beta diversity, we found that *Firmicutes* (*haemolysans* and *micra* species) and *Fusobacteria* (*nucleatum* species) dominated significantly in patients compared to controls. The same phyla (*Firmicutes* and *Fusobacteria*) but different species were reported by the Scaglione et al. study.

Interestingly, there was no significant difference in the microbial composition between smoker patients and non-smoker patients in our study.

In this study, we highlighted the unique malodor of oral PV lesions which has never been reported for another mucocutaneous, bullous disease. Halitosis is an oral health condition characterized by consistently emanating odorous breath. It is, primarily, the result of the degradation of organic substrates by anaerobic bacteria coating the tongue. These microorganisms include mainly not only Gram-negative strains such as *Prevotella melaninogenica*, *Porphyromonas gingivalis*, *Fusobacterium nucleatum (F. nucleatum)*, *Porphyromonas endodontalis*, *Tannerella forsythia*, *Prevotella intermedia*, *Centipeda periodontii*, *Eikenella corrodens*, *Fusobacterium periodonticum* but also some Gram-positive strains such as *Solobacterium moorei*, *Parvimonas micra* (*P. micra*) and *Eubacterium* species (Carlsson et al. 1993; Amou et al. 2014). The result of their action is the production of a range of malodorous molecules mainly volatile sulphur compounds (including methyl mercaptan, hydrogen sulphide and dimethyl sulphide) (Hughes and McNab 2008). Kanwar et al. were the first to focus on this characteristic fishy odor emerging from pemphigus lesions. They pointed that an experienced clinician could recognize this unique odor and even use it as a diagnostic tool. Authors went on to note that this odor is probably due to the colonization of lesions by bacteria. However, the authors did not undertake a research on the origin of this fetor (Kanwar et al. 1992). In the present study, although anaerobic bacterial species were prevalent in the microbiome of all patients, no statistical significance was found compared with the control subjects.

Many of the bacteria responsible for the oral halitosis have simultaneous periopathogenic role. In our research, *F. nucleatum* and *P. micra* were the only periopathogenic dominant species found in patients. The main periopathogenic bacteria which are responsible for initiation and progression of periodontitis are those belonging to the "red complex", *Porphyromonas Gingivalis*, *Tannerella forsythia* and *Treponema denticola.* Furthermore, *A. actinomycetemcomitans* which has been reported as a key pathogen in chronic,

adult periodontitis and in localized aggressive periodontitis was not found in the patients' group either. A cluster of species with less strong association with periodontal inflammation characterized as the "orange complex" includes *Prevotella* spp., *Fusobacterium* spp. and *P. micra* (Costalonga and Herzberg 2014; Fine et al. 2019). In conclusion, the existence of *F. nucleatum* and *P. micra* in our patients did not appear to be related to periodontal disease.

F. nucleatum, a Gram-negative anaerobic oral bacterium, is a main bacterial strain related to halitosis as oftentimes has been mentioned in the literature (Krespi et al. 2006; Scully and Greenman 2012; Hampelska et al. 2020). Aggregation of *F. nucleatum* with other bacteria to form plaque biofilms in oral cavity can cause bad breath. Blocking the bacterial co-aggregation, therefore, may prevent halitosis (Liu et al. 2010, 2013). *P. micra*, a Gram-positive anaerobic oral bacterium, has been linked to oral bad breath sporadically and less frequently (Carlsson et al. 1993; Veloso et al. 2020).

Patients with oral halitosis have a specific biofilm on the dorsal part of the tongue. This is due to the anatomy of the tongue surface which favors low levels of oxygen. The prevailing oxygen conditions in turn lead to the colonization of dorsal tongue by anaerobic bacteria (Seerangaiyan et al. 2018). Bernardi et al. stated that this biofilm consists of a significant proportion of *F. nucleatum* and *Streptococcus* spp. (Bernardi et al. 2019). In the present study, *F. nucleatum* and *P. micra* were found to dominate significantly in PV patients compared to healthy subjects. Moreover, there are studies which have reported that there are specific bacterial species correlated with the absence of intraoral halitosis. Those species mostly associated with fresh breath are *Streptococcus salivarius* and *Rothia mucilaginosa* (Seerangaiyan et al. 2017; Bernardi et al. 2020). Our findings come in accordance with this postulation since both of the aforementioned bacteria were in differential abundance in the healthy group. In the same direction are the results of studies which have showed that administration of probiotic *Streptococcus salivarius* resulted in a prolonged reduction in volatile sulphur compounds (Burton et al. 2006; Benic et al. 2019). Hence, the unique fetor observed in PV patients might be attributed either to the dominance of *F. nucleatum* and *P. micra* and/or the lower levels of *Streptococcus salivarius* and *Rothia mucilanginosa* in oral PV lesions, especially those located on the tongue a common site for disease's manifestation.

An interesting observation emerged from a-diversity analysis was that *Fusobacterium canifelinum* was detected in a limited number of patients (0.33%). These are bacteria found in oral flora of dogs and cats but have also been isolated in purulent bite wound in a human patient. These bacteria are commonly associated with periodontitis in dogs but they have also been associated with systemic infections (Almstah et al. 2003). Their finding in our patients with oral PV lesions could probably be related with the molecular mimicry mechanism of autoimmune reaction.

Research data, including those found from our own study, are not sufficient to determine whether dysbiosis of OMB is the consequence or the cause of autoimmune bullous diseases. Accumulating evidence supports a role of the commensal bacteria in modulation of the immune system which could lead to an autoimmune inflammation. On the other hand, we should keep in mind that PV patients have generally advanced age and a high probability of suffering from periodontitis. Due to painful oral lesions they avoid the daily, routine oral hygiene habits leading to the accumulation of periopathogenic microorganisms. However, in our patient group, we did not find increased levels of main periopathogenic bacteria. Further investigations with satisfactory sample size from large dermatologic clinics or oral medicine centers are mandatory to clarify the exact relationship between OMB and PV or other bullous diseases.

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Author contributions This statement is to certify that Matina Zorba, Angeliki Melidou, Aikaterini Patsatsi, Athanasios Poulopoulos, Georgia Gioula, Alexandros Kolokotronis and Fani Minti have submitted an originally authored article entitled: 'The role of oral microbiome in Pemphigus Vulgaris' to "Archives of Microbiology" for publication. Authors warrant to the Journal and the Publisher that the present article is original, it is not under consideration for another publication and does not infringe trademark, copyright, trade secrets rights or other proprietary rights of third persons (IP rights). Moreover, it has not been published elsewhere in full or partial form, in English or in any other language, including electronically. All authors have made substantial contributions to full steps of this study's completion. They have drafted the work or revised it critically for important intellectual content, approved the final version prior to its submission and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to declare that are relevant to the content of this article.

Ethics approval The ethics committee of School of Dentistry, Faculty of Health Sciences, Aristotle University of Thessaloniki, Greece, confirmed that all the procedures described in this study were carried out in accordance with the principles of the ethics regulation of the research committee regarding the prescribed standard practices for biological research using microorganisms.

Consent to participate and consent for publication Before sampling, each patient was given a written form with details about the title, the

aim of the study, the subjects' selection criteria and participation conditions, detailed description of sampling and research methodology. They were assured that the confidentiality of their personal data would be respected. Furthermore, they became aware that there was a possibility for the results of the study to be published in a scientific journal of a relevant subject. If they would be interested in obtaining a copy of the future published results, they could contact the Department of Oral Medicine and Oral Pathology, Aristotle University of Thessaloniki, Greece. After their detailed information of the above, in case of consent, both the patients (or the person who would receive the card on their behalf) and the researcher signed the form stating the specific date. Signing the consent form the patients declared that (a) they had read and understood the details of the study and they had the opportunity to make questions, (b) they had understood that their participation is voluntary and that they were free to leave at any time they would decide without having to explain the reasons and without their medical care and their legal rights being violated and c) they had understood that some parts of their medical history would be disclosed to certain responsible people and authorities where this would be necessary and gave these people permission to access their history.

Availability of data and materials/code availability Authors make sure that all data and materials as well as software application support their published claims and comply with field standards.

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