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Nasopharyngeal Microbiome Signature in COVID-19-Positive Patients: Can We Definitively Find a Role for *Fusobacterium periodonticum*?

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Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) caused the pandemic Coronavirus Disease 2019 (COVID-19). This virus is highly transmissible among individuals through both droplets and aerosols, and it can lead to severe pneumonia. Among the various factors that can influence both the onset of disease and the severity of its complications, the microbiome composition has also been investigated. Recent evidence showed a possible relationship between the gut, lung, nasopharyngeal, or oral microbiome and COVID-19, but very little is known about it. Therefore, we aimed to verify the relationships between the nasopharyngeal microbiome and the development of either COVID-19 or the severity of symptoms. To this purpose, we analyzed, by next generation sequencing, the hypervariable V1-V2-V3 regions of the bacterial 16S rRNA in nasopharyngeal swabs from SARS-CoV-2-infected patients (n=18) and control (CO) individuals (n=12) using Microbiota solution A (Arrow Diagnostics). We found a significant lower abundance of Proteobacteria and Fusobacteria in COVID-19 patients with respect to CO ($p=0.003$ and $p<0.0001$, respectively) from the phylum up to the genus ($p<0.001$). The *Fusobacterium periodonticum* (*FP*) is the most significantly reduced species in COVID-19 patients with respect to CO. *FP* is reported as being able to perform the surface sialylation. Noteworthy, some sialic acids residues on the cell surface could work as additional S protein of SARS-CoV-2 receptors. Consequently, SARS-CoV-2 could use sialic acids as receptors to bind to the epithelium of the respiratory tract, promoting its clustering and the disease development. We can therefore speculate that the significant reduction of *FP* in COVID-19 patients could be directly or indirectly linked to the modulation of sialic acid metabolism. Finally, viral or environmental factors capable of

interfering with sialic metabolism could determine a fall in the individual protection from SARS-CoV-2. Further studies are necessary to clarify the precise role of *FP* in COVID-19.

Keywords: nasopharyngeal swab, microbiota, next generation sequencing, SARS-CoV-2, *Fusobacterium periodonticum*

INTRODUCTION

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the cause of pandemic Coronavirus Disease 2019 (COVID-19) (Zhu et al., 2020). Most COVID-19 patients show acute respiratory distress syndrome and typical symptoms, including fever, dry cough, and tiredness (He et al., 2020). Other patients suffer from pain, nasal congestion, anosmia, sore throat, or gastrointestinal illness such as diarrhea (Khatiwada and Subedi, 2020). Moreover, a significant number of subjects who test positive for SARS-CoV-2 using a molecular assay, especially during this current wave of the pandemic, are asymptomatic carriers of the virus (Khatiwada and Subedi, 2020). There are many factors (genetic, comorbidities, age, gender) that can influence both the onset of disease and the relative severity of its complications (Abu Hammad et al., 2020; Russo et al., 2020). Among these, the microbiome composition at different levels (gut, lung, skin) was also investigated. The microbiome, the collective genomes of all microorganisms living in our body, in fact, can play a pivotal role in the development of several diseases (Wang et al., 2017; Nardelli et al., 2020; Sacchetti and Nardelli, 2020; Scaglione et al., 2020). In fact, a perturbation of the microbial composition, named dysbiosis, could decrease the microbiota diversity, changing its composition, and promote an inflammatory environment favoring the coronavirus invasion and viral replication (Antunes et al., 2020). The latter induces a strong inflammatory response with the consequent massive release of cytokines and chemokines (the so-called "cytokine storm"). The latter results in a systemic damage, with multiorgan injury, particularly in patients with severe COVID-19 (Abu Hammad et al., 2020). Recent publications showed the possible relationship between the gut, lung, nasopharyngeal, or oral microbiome and COVID-19 (Antunes et al., 2020; Bao et al., 2020; De Maio et al., 2020), but very little is known about it. Therefore, we aimed to verify if the nasopharyngeal microbiome could influence both the development of COVID-19 and the severity of its symptoms. To this purpose, we analyzed the nasopharyngeal microbiome in COVID-19 patients and control individuals in order to investigate the possible association between the microbiome composition and features of COVID-19.

MATERIALS AND METHODS

Patients and Controls

Thirty-eight subjects were included in the study between May and September 2020. These individuals were divided in three groups: n=12 controls (8 females and 4 males; age range: 30–60) who tested negative during a SARS-CoV-2 molecular assay; n=18 symptomatic COVID-19-positive patients (6 females and 12 males; age range: 35–84 years; namely T0) who were submitted to nasopharyngeal swab at admission within the Department of Malattie Infettive, University of Naples Federico II. The severity of the symptoms was evaluated according to the Clinical Status Ordinal Scale as reported by Beigel et al. (2020).

When possible, a second swab was collected 1 week after recovery (T1; n=8 patients). The COVID-19 diagnosis was performed combining the clinical features with the SARS-CoV-2 RNA detection by using a real-time reverse transcriptase-polymerase chain reaction (RT-PCR) assay on the nasopharyngeal swabs. This analysis was performed in the COVID-19 molecular reference Lab n. 777777 of CEINGE Biotecnologie Avanzate S.C.a R.L., belonging to the CORONET Campania Regional network for SARS-CoV-2 diagnostics.

The study was approved by the Ethical Committee of the University Federico II of Naples (authorization n.180/20/ES1 on 25.05.2020). All the enrolled subjects signed the informed consent to participate in the study: our research was conducted in accordance with the Helsinki Declaration policy (2013). The clinical and anamnestic data of each subject, collected by the clinicians, are reported in **Table 1**.

Sample Collection and Storage

For each individual, we collected a dedicated nasopharyngeal swab for the molecular assay using sterile cotton swabs (COPAN SPA, Brescia, Italy). An aliquot of these swabs was stored at -80° C until used. The swab sample was drawn at T0 before starting any drug treatment.

DNA Isolation

All swab samples were thawed at room temperature, and bacterial DNA was isolated using MagPurix® Bacterial DNA Extraction Kit (Zinexts Life Science, New Taipei City, Taiwan), according to manufacturer instructions. All extractions were performed in a pre-PCR-designated room in a COVID laboratory (CEINGE). DNA samples were stored at −20°C until further processing. The yield and the quality of the extracted DNA were determined using a Qubit dsDNA HS (High Sensitivity) assay kit (Invitrogen Co., Life Sciences, Carlsbad, USA) run on the TapeStation (Agilent Technologies, Santa Clara, CA, USA).

16S rRNA Sequencing and Data Analysis

The hypervariable V1-V2-V3 regions of the bacterial 16S rRNA were amplified using Microbiota solution A (Arrow Diagnostics, Genova, Italy) according to the manufacturer instructions. The quality and quantity of amplification products were evaluated by TapeStation system and Qubit dsDNA BR assay. This step is

TABLE 1 | General and biochemical characteristics of n=18 COVID-19 patients.

Parameters	T0	T1
Age range (years)	35–84	
Gender	6 F/12M	
Weight (Kg)	75.5 ± 16.4	
Height (m)	1.7 ± 0.03	
Red blood cells (U/L)	4306250 ± 854416	3915000 ± 568984
White blood cells (U/mL)	7438.75 ± 4557.9	7776.2 ± 3573.6
Neutrophils (U/mL)	5675 ± 4105	4481.3 ± 2525.3
Lymphocytes (U/mL)	1238.75 ± 652.8	1472.5 ± 512.6
Hemoglobin (g/mL)	12.3 ± 2.2	11.0 ± 1.4
Platelets (U/mL)	273250 ± 227449	234875 ± 137843
Aspartate aminotransferase (U/L)	25.3 ± 12.3	28.25 ± 8.1
Alanine aminotransferase (U/L)	15.5 (11.7–31.0)	18.5 (13.2–41.7)
Creatinine (mg/dL)	1.2 ± 1.0	1.1 ± 1.0
Azotemia (mg/dL)	50.6 ± 34.7	53.4 ± 39.4
Creatine Phosphokinase (U/L)	24 (15.7–38.7)	40.5 (28.5–79.7)
pro-BNP* (pg/mL)	74.3 ± 62.8	72.4 ± 51.8
Prothrombin time (s)	1.1 ± 0.08	1.1 ± 0.08
Partial thromboplastin time (s)	1.0 ± 0.1	1.0 ± 0.1
D-Dimer (ng/mL)	2.0 ± 1.6	2.2 ± 1.6
Fibrinogen (mg/mL)	476.5 ± 118.2	449.6 ± 134.3
Reactive C protein (mg/L)	4.1 (1.4–9.9)	2.0 (0.4–4.3)
Inteleukin-6 (pg/mL)	21.1 (9.0–29.8)	9.9 (5.5–20.2)
Ferritin (ng/mL)	248.5 (214.2–423.2)	264.5 (131.5–597.7)

Data are represented as mean ± standard deviation or median (25°–75° percentiles) for non-parametric variables.
**N-terminal prohormone of brain natriuretic peptide.*

preliminary to the pooling procedure consisting of equimolar libraries. Sequencing of our libraries was performed on MiSeq Illumina® sequencing platform (Illumina, CA, US) using V2 500 cycles reagent. Sequencing data were analyzed by dedicated bioinformatics software (MicrobAT Suite - SmartSeq, Novara, Italy) that could decipher the operational taxonomic units (OTUs). Statistical analyses were performed by MicrobiomeAnalyst program (https://www.microbiomeanalyst.ca/) using default parameters. The alpha diversity was evaluated using the Chao-1, Shannon, and Simpson indices, respectively. Wilcoxon rank-sum test (Mann-Whitney) was performed to test the significance of pairwise richness differences. The beta-diversity has been evaluated through non-metric multidimensional scaling (NMDS) ordination of variance, which stabilized counts of taxa for CO and COVID positive samples, and compared using Bray-Curtis dissimilarity. Permutation analysis of variance (PERMANOVA) and corresponding R-squared and p-values were calculated. By Kruskal-Wallis Rank Sum Test, the abundance of taxa, at each taxonomic level, were compared. Comparisons were performed between either CO and COVID+ or CO and COVID+ (at T0 and T1 of observation). The Raw and processed high-throughput sequencing data have been deposited in the Sequence Read Archive (SRA) (https://www.ncbi.nlm.nih.gov/sra) under Project SUB8444038.

RESULTS

The sequencing of all nasopharyngeal swabs produced an average of 67,500 counts per sample.

The nasopharyngeal microbiome was different in patients with COVID-19 (T0) with respect to control subjects (CO). Alpha diversity was evaluated through Chao1, Shannon, and Simpson diversity indices, the latter measuring the within-sample diversity and comparing species richness between the analyzed groups. All indexes were shown to not be statistically different to comparisons between COVID-19 patients and CO (Chao1: p=0.28, Shannon: p=0.27, and Simpson: p=0.32) (**Figure 1**).

The beta diversity was measured by Bray-Curtis (p=0.007) and Jaccard (p=0.006) analysis, *via* the PCoA ordination method, for the evaluation of dissimilarities in patients and controls (**Figure 2**). Our analysis confirmed that the distance between groups was dependent on the relative abundance of taxa rather than on the type (**Figure 2**).

Taxonomic assignment indicated that the nasopharyngeal microbiome in CO and COVID-19 (T0 and T1) individuals consisted of five distinct phyla: Firmicutes (CO = 43.7%, T0 = 49.5%, and T1 = 55.0%), Bacteroidetes (CO = 21.6%, T0 = 24.0%,

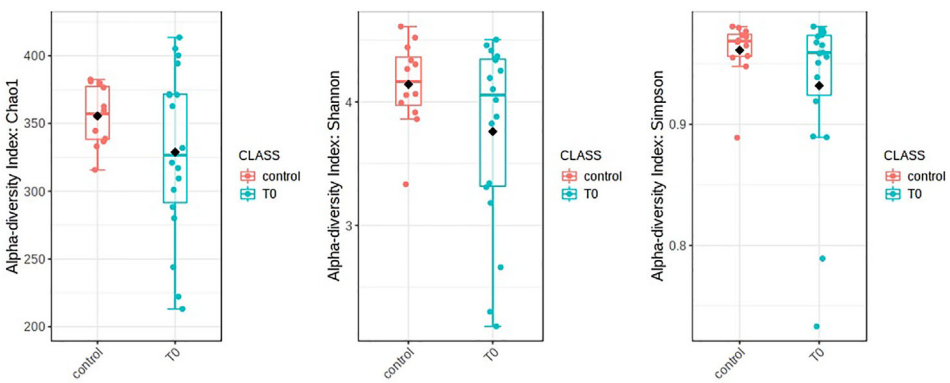

FIGURE 1 | Alpha diversity in patients with COVID-19 (T0) in respect to control subjects. Alpha diversity analysis was evaluated by means of several metrics: Chao1, Shannon, and Simpson indices. Overall, the plots do not show any significant difference between T0 and Control (statistical analysis by Wilcoxon rank-sum test Mann-Whitney). Nevertheless, a soft trend of decreased richness in T0 respect to Control was observed.

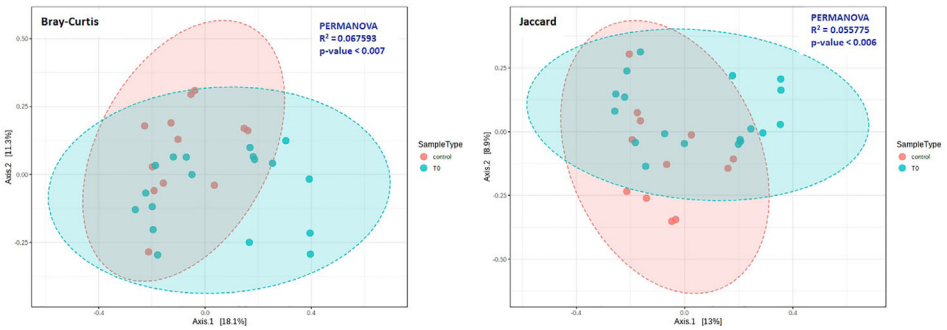

FIGURE 2 | Beta diversity in COVID-19 (T0) and Control groups. Principal coordinate analysis (PCoA) plots were performed by the Bray-Curtis (left) or the Jaccard (right) distance measures. Statistical significance was assessed by PERMANOVA. In both cases we found a significant result (Bray-Curtis: p = 0.007; Jaccard: p = 0.006), and analysis confirmed that the distance between groups was dependent on the relative abundance of taxa rather than on the type.

and T1 = 28.6%), Actinobacteria (CO = 8.9%, T0 = 14.5%, and T1 = 9.1%), Proteobacteria (CO = 13.8%, T0 = 7.4%, and T1 = 5.0%), and Fusobacteria (CO = 10.6%, T0 = 3.2%, and T1 = 1.7%) with a relative abundance >1% in all groups (**Figure 3**).

We found a significant lower abundance of Proteobacteria and Fusobacteria in T0 and T1 with respect to CO (p=0.003 and p<0.0001, respectively). The significant reduction of these phyla among two groups was evaluated from the class up to the genus (p<0.001) by using the Kruskal-Wallis test (**Figures 3A–F**). At the genus level, we found a significantly reduced relative abundance of *Leptotrichia, Fusobacterium*, and *Haemophilus* in T0 compared to CO (p= .01, 0.002, and 0.001, respectively) (**Figure 3E**). When the species were considered, the *Fusobacterium periodonticum* (*FP*) was significantly reduced in COVID-19 patients (both at T0 and T1 sampling) compared to CO subjects (p<0.01) (**Figure 3F**).

No statistical differences were observed when we compared the T0 vs T1: in **Figure 3A**, the relative abundance of the T1 group is reported in relation to the phylum level. In the **Supplemental file** the relative abundance (%) of all taxonomic levels from Phylum to Species, obtained by using the MicrobAT Suite – SmartSeq, is reported.

Interestingly, despite the limited number of analyzed patients, we observed a negative correlation between the relative abundance of *Fusobacterium Periodonticum* and the severity of the patients' symptoms (p=0.034, R^2 = 0.25, R=-0.5).

DISCUSSION

To date, little is reported about the relationship between the microbiome and SARS-CoV-2 infection, particularly due to the difficulties in obtaining peculiar samples in severely symptomatic patients within the emergency departments or intensive care units. So, the standardization of procedures surrounding these types of studies can be biased by preanalytical variables (time of sample drawing, types of patients, personnel operating swabs, etc.) (Kim et al., 2017). Nevertheless, the microbial composition plays a very important role as an indicator of either healthy or disease status (Wang et al., 2017). Several studies investigated if the microbiota could modify the risk of developing COVID-19 by evaluating its complications particularly at respiratory level (Bao et al., 2020; De Maio et al., 2020; He et al., 2020; Khatiwada and Subedi, 2020; Shen et al., 2020). The recent evidence reported that some microorganisms are associated with SARS-CoV-2 infection. In particular, Wu et al. found *Leptotrichia buccalis, Veillonella parvula, Capnocytophaga gingivalis,* and *Prevotella melanogenica* as overexpressed in bronchoalveolar lavage liquid (BALF) of the COVID-19 patients (Wu et al., 2020); Budding et al. reported an association between the composition of the pharyngeal microbiota and SARS-CoV-2 infection, highlighting a less diverse microbial profile in older individuals. This finding could explain the enhanced susceptibility of the elderly subjects to SARS-CoV-2 infection (Budding et al., 2020).

In our study, we found a different microbial composition in nasopharyngeal swabs between COVID-19 patients and controls. Noteworthy, COVID-19 patients showed a significant reduced abundance in Proteobacteria and Fusobacteria as compared to controls; this abundance was confirmed at every taxonomic level of microbiota analysis (from phylum to species). In fact, we found a significant reduction in the *Leptotrichia* and *Haemophilus* genus: these findings cannot be considered as superimposable to those reported by Wu et al. since they analyzed broncho-alveolar lavage fluids (BALFs). In this regard, we underline the recovery of BALF is very prone to pre-analytical variability (Pocino et al., 2015), and, therefore, the comparison of data obtained from different research groups is sometimes challenging.

However, by analyzing the microbial content of our patients and controls, we found that *FP* was the most represented in the CO group contrary to the COVID-19 patients. The role of the *FP* is yet unknown and, if it could regulate the ACEs expression, as shown for other intestinal microbes in COVID-19 patients (Geva-Zatorsky et al., 2017), is still to be clarified. Our findings are in agreement with those by Moore et al. who reported that *FP* was shown to be significantly decreased 3 days following SARS-CoV-2 infection (Moore et al., 2020). Our data show that a progressive decline in this species was evident on swabs collected 1 week after admission to

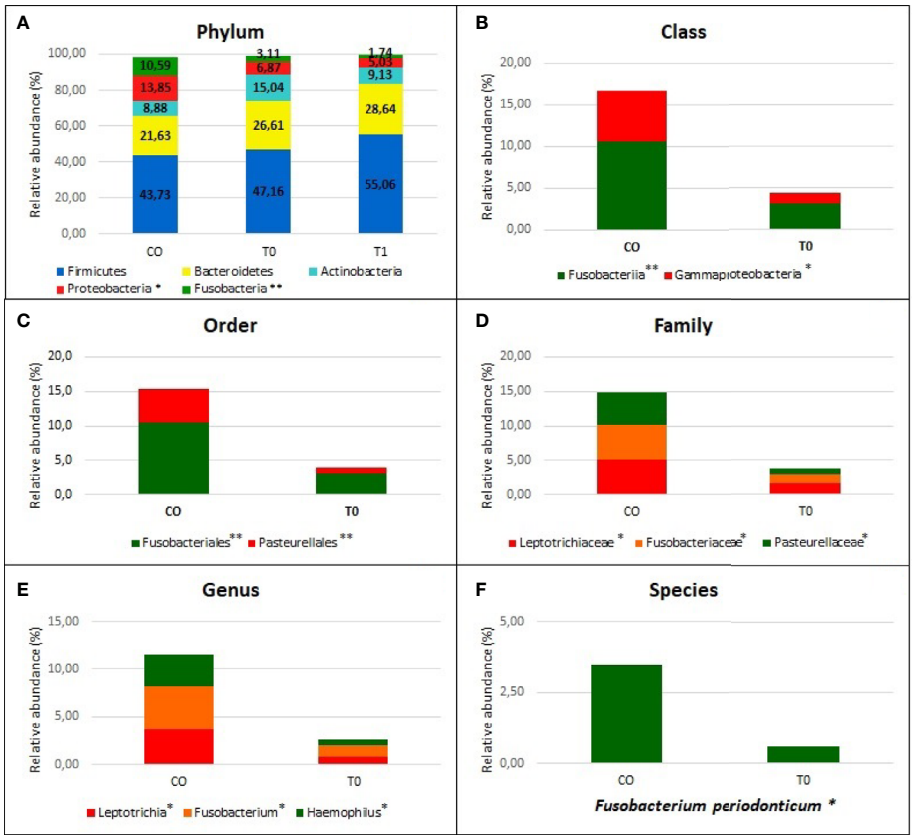

FIGURE 3 | Nasopaheryngeal microbiome composition in COVID-19 patients and Control group. The graphs show the percentage of relative abundance (%) of the all taxonomic levels from Phylum to Species, obtained by using the MicrobAT Suite - SmartSeq. Each column in the plot represents a group, and each color in the column represents the relative abundance (%) for each taxon. In panel **(A)** we show the phyla with average relative abundance greater than 1% in all studied groups; we found two phyla significantly less abundant in COVID-19 patients with respect to Controls, Proteobacteria, and Fusobacteria. Not statistically significant difference in taxa abundance was observed when T0 and T1 COVID-19 patients were compared. The other panels **(B–F)** show the taxa abundance from class to up species level significantly different between groups by Kruskal Wallis test. **(B)** class **(C)** order, **(D)** family, **(E)** genus **(F)** species. $^{*}p < 0.05$; $^{**}p < 0.001$.

hospital. Interestingly, Yoneda et al. reported that *Fusobacterium periodonticum* is involved in the surface sialylation process (Yoneda et al., 2014). Morniroli et al. reported that some sialic acid residues on the cell surface could work as alternative receptors of the SARS-CoV-2 S protein, in addition to the ACE2, influencing the development of the associated disease. Unfortunately, we cannot currently provide any evidence of a functional relationship (who drives who?) in this regard. Both Mormiroli and Yoneda (Yoneda et al., 2014; Morniroli et al., 2020) have reported that the sialome also plays a defensive role against viral infections, where a reduction of sialic metabolism can reduce the protection of individuals from SARS-CoV-2.

We point out Fusobacteria exhibit strong adherence to numerous human cell types, probably influencing the modulation of the host inflammatory response: this aspect could explain our findings, showing a negative correlation between the relative abundance of *Fusobacterium Periodonticum* and the severity of symptoms. This result could strengthen our hypothesis of a potential protective role of *FP* against SARS-CoV-2. We have planned to deepen this functional aspect in coming experimental settings that, unfortunately, take time, *FP* being a slow growing microorganism.

Our study presents the following strengths: (1) our COVID-19 patients were all enrolled within the same Department; (2) the nasopharyngeal swab collection was always performed using a standardized procedure from the same selected medical staff to reduce pre-analytical biases; (3) the nasopharyngeal swab collection was performed upon admission for all patients with diagnosis of COVID-19, confirmed by molecular assay, before starting with any drug treatment; (4) the comparison between COVID-19-positive patients and a cohort of healthy controls regarded individuals coming from the same geographical region, reducing the environment-dependent variability; (5) the control group were all negative to SARS-CoV-2 infection, as they were all recruited from healthy people working in our hospital, who are periodically monitored with molecular assay, in compliance with our hospital policy.

Limitations of our study: one of the limitations of this work is represented by small number of patients and controls analyzed. Nevertheless, due to difficulties related to the enrollment of

COVID-19 patients, also other similar studies were not able to collect greater cohorts of subjects. Moreover, although our study analyzed a limited number of patients, our patients and controls were all clinically staged by an *ad hoc* medical team at admission and were also evaluated in the follow-up, when possible. Further studies are needed to confirm these data on larger patient cohorts. Also, functional *in vitro* studies are ongoing to decipher the molecular mechanisms of interaction between *FP* and SARS-CoV-2.

We also highlight that De Maio et al. (2020) stated that SARS-CoV-2 did not modify the microbiome composition of the patients as compared to the controls: we believe that the lack of any statistical difference reported could be due to the different characteristics of patients enrolled (mainly with mild disease form).

Finally, although the perfect layout for studying microbiota is still challenging to realize, we can consider our findings as the first clearly showing a potential role of Fusobacteria in protecting oral mucosa from SARS-CoV-2 infection. Further research will be performed to define the pathophysiological mechanisms of this apparently protective effect of *Fusobacterium periodonticum* against COVID-19.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/sra, SUB8444038.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical Committee of the University Federico II of Naples (authorization n.180/20/ES1 on 25.05.2020). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CN: execution of experiments, data and statistical analyses, and manuscript writing. IG, BP, and CD: patient recruitment and sample and clinical data collection. MS: technical collaboration to part of experiments. GS: Bioinformatic support. GC: Elaboration and revision of ethical committee forms and documentation. EC: study conception and design, final revision, and editing of the manuscript. All authors have read and agreed to the published version of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.625581/full#supplementary-material

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Evaluation of cutaneous, oral and intestinal microbiota in patients affected by pemphigus and bullous pemphigoid: A pilot study

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ABSTRACT

Background: Significant alterations of the cutaneous microbiota (CM) have been recently demonstrated in bullous pemphigoid (BP). Microbiome data of both oral cavity (OM) and gut (GM) from patients affected by bullous disease are not available yet and, further consistent studies focused on the role of such microbial populations are still missing.
Objective: Objective: In this pilot study we characterized and compared GM, OM and CM of patients affected by pemphigus vulgaris (PV) and BP to investigate a distinctive microbiome composition in this two rare dermatological disorders.
Methods: High-throughput sequencing of the V1-V3 hyper-variable regions of 16S rRNA was used to compare the bacterial community composition of stool, skin and oral mucosae swabs in a cohort of PV and BP patients. A dedicated bioinformatics software coupled with *in-house* pipeline was implemented to analyse and compare diseases dataset.
Results: GM samples of both PV and BP patients were principally characterized by Firmicutes and Bacteroidetes phyla. Interestingly, the Firmicutes phylum and *Staphylococcus* genus were mainly represented in cutaneous samples. The diversity of phyla in oral mucosae was higher than those of gut and skin samples and, Bacteroidetes phylum was significantly underrepresented in all PV samples.
Conclusion: Firmicutes phylum and *Staphilococcus* genus were the most represented in OM and CM swabs of PV and BP microbial populations. Moreover, we argue the quantitative imbalance linked to the decrease of Bacteriodetes in the oral cavity of PV patients might be associated to disease typical fetor. To shed light on this peculiar feature further studies are still required.

1. Introduction

The most important autoimmune bullous diseases are Pemphigus and Bullous Pemphigoid that are rare and life-threatening diseases provoked by Autoantibodies (Abs) directed against components of the skin and mucosae (Schmidt and Zillikens, 2010). Particularly, in pemphigus patients, Abs bind components of intercellular desmosomes, named Desmoglein (Dsg) 1 and 3 (Di Zenzo et al., 2017); two major

Abbreviations: BP, Bullous Pemphigoid; Abs, Autoantibodies; Dsg, Desmoglein; PV, Pemphigus Vulgaris; PF, Pemphigus Foliaceus; CM, Cutaneous Microbiota; EBA, Epidermolysis Bullosa Acquisita; OM, Oral Microbiota; GM, Gut Microbiota; IBD, Inflammatory Bowel Disease; NGS, Next Generation Sequencing; HMP, Human Microbiome Project; OTU, Operational Taxonomic Unit; FISH, Fluorescence in situ hybridization; RFLP, Restriction Fragment Length Polymorphism; PFGE, Pulsed-Field Gel Electrophoresis; AD, Atopic Dermatitis; LES, Systemic Lupus Erythematosus

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types of pemphigus have been described that are Pemphigus Vulgaris (PV) and Pemphigus Foliaceus (PF) characterized by mucous and/or cutaneous lesions associated with IgG Abs against Dsg 3 and Dsg1, respectively (Di Zenzo et al., 2016). In BP patients, Abs are directed against two hemidesmosomal components: BP180 and BP230 (Di Zenzo et al., 2012).

The human skin is a critical interface with the external ambient and provides a range of niches colonized by microbes that overall form the cutaneous microbiota (CM). Imbalance or shift in the homeostasis between microbial composition and host at a given body site (dysbiosis) has been widely associated with several diseases. Particularly, the interaction of the CM, host and environment is closely related to the human wellness and/or to the outbreak of diseases (Rosenthal et al., 2011; Grice et al., 2008; Schommer and Gallo, 2013). Recent studies affirm that host immunity could be influenced by the CM and, on the contrary, the CM partially modulates cutaneous immunity (Nestle et al., 2009; Chehoud et al., 2013). At least nineteen phyla are known as being a part of the healthy bacterial skin microbiota. Main examples are the Actinobacteria, Firmicutes, Proteobacteria and Bacteroidetes. Across the different skin regions, the abundance of each group is strongly dependent on the characteristics of the appropriate niche. For example, in sebaceous sites *Propionibacterium* and *Staphylococcus* are preponderant while both *Corynebacterium* and *Staphylococcus* are found in moist area. In contrast, mixed populations of bacterial species are present in cutaneous dry sites (Schommer and Gallo, 2013; Nestle et al., 2009; Chehoud et al., 2013; Chen et al., 2018a). The composition of the CM can shift markedly during inflammation. Two recent studies investigated the role of CM in two bullous diseases, i.e. epidermolysis bullosa acquisita (EBA) and BP. Ellebrecht et al. studied the CM involvement in EBA development after immunization of SJL/J mice. The authors concluded that the mechanism surrounding the formation of the blisters in the presence of Abs is depending on the skin rather than the lymph nodes, and that a greater amount of cutaneous bacterial species appears to be protective. The authors suggested also that a subclinical cutaneous inflammation, which is associated with skin microbial diversity, strongly influences the manifestation of the disease (Ellebrecht et al., 2016). Moreover, Miodovnik et al. demonstrated that there is a significant difference in CM between BP patients and healthy controls. They also suggested that modulation of the CM could be a potential therapeutic strategy (Miodovnik et al., 2017).

Similarly, as one of the most clinically relevant human sites, also the oral cavity is colonized by a set of microorganisms, including bacteria, fungi and viruses. Compared to other oral niches (e.g. dental plaque), microbial species colonizing the oral mucosae are relatively limited. A relatively small number of bacterial phyla, generally belonging to Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria, and Fusobacteria (Aas et al., 2005; Schmidt et al., 2014; Keijser et al., 2008; Zaura et al., 2009; Nasidze et al., 2009a; Nasidze et al., 2009b; Bik et al., 2010) characterizes healthy oral microbiota (OM). Within the healthy oral cavity, most habitats are dominated by *Streptococcus*, followed in abundance by *Haemophilus*, *Prevotella*, *Veillonella* and *Neisseria* (Aas et al., 2005; Schmidt et al., 2014; Keijser et al., 2008; Zaura et al., 2009; Nasidze et al., 2009a; Nasidze et al., 2009b; Bik et al., 2010). Increasing evidence has supported that this OM contribute to the two commonest oral diseases of man (dental caries and periodontal disease) (He et al., 2015). Notably, in the microbial community composition of biofilms could be present a difference at each intraoral habitat, like in the tooth surface or in the lateral and dorsal tongue due to the different surface properties and microenvironments (Zaura et al., 2009; Bik et al., 2010). Among these sites, the tongue has attracted great attention due to its colonization by microbes associated with halitosis, the latter a typical feature of PV patients.

In addition to skin and oral cavity, also the gut microbiota (GM) was deeply investigated by other groups. In detail, four different phyla (Bacteroidetes, Actinobacteria, Firmicutes and Proteobacteria), consisting of thousands of mostly anaerobic species, reside in the human gut (Konturek et al., 2015). According to the multiple physiological functions of the intestine, it is not surprising that the impairment of its bacterial composition is involved in gastrointestinal as well as non-gastrointestinal diseases (e.g. obesity/metabolic syndrome, atherosclerosis/cardiovascular, neurologic/psychiatric diseases)(Blum, 2017). Regarding the inflammatory bowel disease (IBD), the beneficial bacteria could protect the patient from inflammation, conversely, pathobionts favour the inflammation. Several studies reported an alteration of GM also in Crohn's disease, particularly related to a decrease of Firmicutes abundance and a simultaneous increase of Proteobacteria and Bacteroidetes phyla. Particularly, lower levels of *Faecalibacterium prausnitzii* is a well-established replicated results in gut microbial composition of affected patients when compared to healthy controls (Zaura et al., 2009; Fujimoto et al., 2013).

Next Generation Sequencing (NGS) targeting the phylogenetically informative 16S ribosomal RNA gene (16S rRNA) is considered as a robust method used in compositional studies of the microbiome, in both healthy and disease status (Srinivas et al., 2013; Peterson et al., 2009; Kong, 2011; Chiller et al., 2001). In this pilot study, high-throughput sequencing approach by NGS was used for the characterization and the evaluation of the CM, OM and GM in patients affected by PV and BP. To the best of our knowledge, this is the first study concerning the analysis of microbial composition of three different body sites involving PV and BP patients.

2. Methods

2.1. Study group

Twelve PV and eight BP patients from the Departments of Dermatology at Istituto Dermopatico dell'Immacolata (IDI)-IRCCS, were included in the study between January and June 2018. The patients satisfied the typical clinical and immunopathological findings of pemphigus and BP: 1) the deposition of IgG/C3 at the intercellular area (pemphigus) or at dermal-epidermal junction (BP) by direct immunofluorescence microscopy; 2) circulating Abs labeling with an intercellular pattern (pemphigus) or at dermal-epidermal junction (BP) by indirect immunofluorescence microscopy; 3) the presence of Abs anti Dsg 1 or 3 in pemphigus, abs against BP180 and 230 in BP, by ELISA. The Ethics Committee of the Istituto Dermopatico dell'Immacolata (IDI)-IRCCS approved the study. All subjects signed a written informed consent and, the study was conducted in accordance with the Declaration of Helsinki. The subjects were all Caucasians, pemphigus cohort included 6 females and 6 males (age: 38–80 yrs.; average: 55 ± 14 yrs). The BP enrolled subjects were 5 females and 3 males, (age: 45–89 yrs.; average: 70 ± 18 yrs) (Table 1). All the subjects did not intake systemic antibiotics, probiotics and lactic ferments for at least one month before enrolment and, topical products for two days before skin sampling were banned as well. Subjects undergoing mucosae swab test avoided the oral hygiene and fasted for 3 h before sampling. Furthermore, it has to be specified that all patients were treated with systemic therapy when samples were taken and this therapy has been indicated in Table 1 as “current dermatologic therapies”.

2.2. Sample collection and storage

For each study participant, up to three different sample types were collected by the Division of Dermatology of the Istituto Dermopatico dell'Immacolata (IDI)-IRCCS. The oral cavity (n = 7, PV) and the skin (n = 3, PV; n = 3, BP) swabs were obtained from the lesional sites, eroded for the cutaneous/mucosal lesions, by swabbing the areas vigorously for 30s using sterile cotton swabs (COPAN SPA, Brescia, Italy), each device was previously moistened with sterile solution (0.15 M NaCl and 0.01% Tween 20). The head of each swab was aseptically cut from the handle into a 2 ml cryovials containing 1 ml specimen

Table 1
Descriptive outline of the subjects enrolled in this study.

Case N°	Sex/Age	Diagnosis	Involved Cutaneous/ Mucosal areas	Ab anti desmoglein 1 and 3/ BP180 and 230	Dermatologic therapies		BPDAI/ PDAI	Sample type		
					Previous	Current		CS	OS	FS
1	M/48	PV	M/C	Dsg1: 140 Dsg3: 36.5	CCS, MTX, IG	CCS, RTX	18	Y	Y	Y
2	F/46	PV	M	Dsg1: 22.7 Dsg3: 137	CCS, RTX	CCS, RTX	3	N	Y	Y
3	F/80	PV	M/C	Dsg3: 168.6	CCS	CCS	14	N	N	Y
4	M/56	PV	C	Dsg1: 91.3	CCS	T-CCS, RTX	15	Y	N	Y
5	F/39	PV	M	Dsg1: 73.3 Dsg3: 25.4	CCS, RTX	CCS, RTX	5	N	Y	N
6	F/77	PV	M/C	Dsg3: 54.8	RTX, AZT	CCS	4	N	Y	Y
7	M/43	PV	M	Dsg1: 168 Dsg3: 200.9	CCS	CCS	2	N	N	Y
8	F/51	PV	M	Dsg1: 18.1 Dsg3: 256	CCS, AZT, MMF	CCS, RTX	12	N	Y	N
9	F/57	PV	M	Dsg3: 157	CCS, RTX	CCS, RTX	9	N	Y	Y
10	M/55	PV	C	Dsg1: 89.6 Dsg3: 95.1	CCS	RTX	2	N	Y	Y
11	M/38	PV	C	Dsg1: 132.5	CCS	T/S CCS	3	N	N	Y
12	M/65	PV	C	Dsg3: 10.3	CCS	T/S CCS	2	Y	N	Y
13	F/84	BP	C	BP180: 126.9 BP230: 85.6	CCS, AH	T/S CCS, AH	48	N	N	Y
14	F/61	BP	M/C	BP180: 46.2	CCS, AH DAPSONE, MMF, IG, RTX	T/S CCS, AH	27	N	N	Y
15	F/83	BP	M/CC	BP180: 52.7	CCS, AH	T/S CCS, AH	15	Y	N	Y
16	M/45	BP	C	BP180:82.1	CCS, AH	T/S CCS, AH	41	N	N	Y
17	M/88	BP	CC	BP180: 74.2 BP230: 34.7	CCS, AH	CCS, AH	8	Y	N	Y
18	F/89	BP	MM	BP180: 22.3	CCS	CCS	1	N	N	Y
19	M/47	BP	CC	BP180: 163 BP230: 74	CCS, AH	CCS, RTX, AH	44	Y	N	N
20	F/66	BP	CC	BP180: 44 BP230: 57.2	CCS, AH	CCS, AH	15	N	N	Y

Footnotes. M: male; F: female; BP: Bullous Pemphigoid; PV: Pemphigus Vulgaris; NA: Not Applicable; Y: yes; N: not; C: Cutaneous; M: Mucosal; Dsg: desmoglein; MTX: Methotrexate, MMF: Mycophenolate Mofetil, Ig: Immunoglobulin, RTX: Rituximab, AH: Antihistamines, AZT: Azathioprine; CCS: Systemic Corticosteroid, T/S CCS: Topical and Systemic Corticosteroids; T-CCS: Topical Corticosteroid; BPDAI: Bullous Pemphigoid Disease Area Index; PDAI: Pemphigus Disease Area Index; CS: Cutaneous Swab; OS: Oral Swab; FS: Fecal Sample.

collection fluid (Tris-EDTA and 0.5% Tween 20) and stored at −80 °C until processing. A set of clean swabs were used as negative controls and they were handled according to the abovementioned protocol. To note, due to the lack of mucosae lesions in BP subjects, we identified and collected a control dataset from the HMP (Human Microbiome Project) database (Schmidt et al., 2014). The stool samples (n = 10, PV; n = 7, BP) were self-collected by patients into a provided commercial sterile stool collection kit, which were stored on ice until transport to the lab (within 24 h from the collection). When delivered to the laboratory, stool samples were splitted into 300-mg samples in sterile cryovials and frozen at −80 °C.

2.3. Sample processing

(i) Swab samples were thawed at room temperature and bacterial DNA was extracted immediately. DNA extraction was performed using QIAamp® DNA Microbiome kit according to manufacturer instructions (Qiagen, Hilden, Germany) with the following modifications: after the incubation of the sample with the provided Buffer AHL, the subsequent centrifugation step was performed at 12,000 x g; in the final elution of the extracted DNA, a total volume of 40 µl of Buffer AVE was applied. The remaining steps were performed as recommended by the manufacturer. DNA samples were stored at −20 °C until further processing.

(ii) Stool sample were thawed and bacterial DNA was extracted immediately. DNA extraction was performed using QIAamp® PowerFecal® DNA kit according to manufacturer instructions (Qiagen, Hilden, Germany). DNA samples were always stored at −20 °C until further processing.

2.4. Evaluation of DNA quantity and quality

The final yield of extracted DNA was determined fluorometrically using Qubit dsDNA HS (High Sensitivity) assay kit on Qubit® Fluorometer 3.0 (Invitrogen Co., Life Sciences, Carlsbad, USA). The integrity of DNA was determined by visualizing 3 µl of the extracted DNA on a 1% agarose gel containing SYBR TM Safe DNA Gel Stain (Invitrogen Co., Life Sciences, Carlsbad, USA).

2.5. 16S rRNA sequencing and data analysis

The amplification of the hypervariable V1-V2-V3 regions of the bacterial 16S rRNA was performed according to manufacturer instructions using Microbiota solution A (Arrow Diagnostics, Genoa, Italy): the libraries obtained were then sequenced on a MiSeq Illumina® sequencing platform (Illumina, CA, US) using V2 500 cycles reagent. Sequencing data were processed, aligned and clustered into operational taxonomic units (OTUs) using a dedicated bioinformatics pipeline (SmartSeq, Novara, Italy).

2.6. Statistical analysis

Statistical data analysis of the OTUs results was performed using STATA software (StataCorp. 2011. Stata Statistical Software: Release 12. College Station, TX: StataCorp LP) applying a threshold of > 5% and > 3% of abundance at the phylum and species phylogenic ranks, respectively. Statistical evaluation was applied to assess the qualitative (presence/absence of OTUs) and quantitative (richness, abundance expressed as the number of reads) parameters. Data were analysed by

Mann-Whitney non-parametric test applying a significance cut-off of $p < .05$.

3. Results

3.1. Statistical analysis of microbial composition

A total number of about 1.69 millions of 16S rRNA sequences were obtained by NGS analysis of 30 samples ($n = 6$ skin, $n = 17$ stool and $n = 7$ oral mucosae samples). The average values of the reads-pairs collected among the sampling site dataset were 2700 ± 200 in stools samples, 11,000 ± 4000 in skin swabs and 4000 ± 700 in oral mucosae swabs. In order to determine the number and abundances of different bacterial groups in each sample, a > 97% threshold of similarity between 16S rRNA gene sequences was used as an indicator of the species level (OTUs). Comprehensively, we found 2058 different OTUs in 30 samples within the three different sampling sites. Finally, data obtained from the OTUs tables were analysed to assess the richness, the evenness and the diversity of the microbial community of the different samples.

3.2. Phyla evaluation

The microbiome analysis at phyla level is reported in Fig. 1. We reported only abundances above 5% as arbitrary cut-off. The relative abundances (%) of the prevalent (> 5%) phyla in the gut (Fig. 1, a), skin (Fig. 1, b) and oral mucosae (Fig. 1, c) groups of samples were plotted for both PV and BP patients. In the case of oral mucosae, the comparative analysis was performed between pemphigus patients and reference controls (Control).

The analysis of intestinal microbiome (Fig. 1, a) shows an overlapping distribution of bacterial phyla in both PV and BP groups. Specifically, the p50 (min-max) of Firmicutes relative abundance (%) was 43.3 (31.8–75.0) in PV and 47.7 (38.8–65.3) in BP patients; Bacteroidetes relative abundance (%) was 50.9 (20.5–66.6) in PV and 43.8 (33.0–50.9) in BP patients, while Proteobacteria resulted in a p50 of 10.3 (5.7–21.4) in PV and 7.8 (5.2–12.7) in BP patients, respectively. The analysis of skin swabs samples (Fig. 1, b) highlighted that Firmicutes phylum was the most abundant, with a relative abundance (%) of 82.4 (82.1–83.2) in PV and 99.3 (55.7–99.9) in BP subjects. Actinobacteria phylum was found in all PV samples (p50 = 17.4; min = 15.8; max = 17.5) while it was present in only one BP subject (p50 = 30.7). Also Proteobacteria phylum was observed in only one BP skin swab sample (p50 = 13.4). Finally, in order to analyse the oral cavity dataset of our PV samples, we first selected a reference control group from the HMP database[14]. Particularly, the abundances of the phyla in five oral swab control samples (region V1-V3) were compared to the seven samples collected in our study. The oral mucosae samples (Fig. 1, c) showed a qualitative higher diversity in bacterial phyla composition when compared to skin swabs and gut. Particularly, we found: (i) Firmicutes(%) = 45.5 (27.1–72.6) in PV and 39.6 (32.3–73.4) in Control samples; (ii) Fusobacteria(%) = 28.0 (10.4–41.6) in PV and 8.5 (1.9–13.2) in Control samples; (iii) Bacteroidetes (%) = 7.2 (5.7–12.6) in PV and 28.4 (7.3–38.5) in Control samples; (iv) Proteobacteria (%) = 15.2 (5.1–23.9) in PV and 13.3 (10.5–42.6) in Control samples; (v) Actinobacteria(%) = 5.5 (2.8–27.0) in PV and 2.4 (1.4–5.3) in Control samples.

Finally, Mann-Whitney test was performed to evaluate our results. Even if no statistical significance was observed in the phyla composition (> 5%) of stool and skin datasets of PV and BP groups, we found that Bacteroidetes phylum was significantly underrepresented ($p = .0112$) in the oral mucosae swabs of PV patients (Fig. 1, c).

3.3. Species evaluation

The qualitative evaluation of microbial composition at the species level is described in Fig. 2. Data represented in stacked columns reflect the relative abundances (%) of each of the 10 most prevalent species identified by NGS assay in the two groups of patients at the different sampling sites. As expected, the analysis of GM (Fig. 2, a) revealed a higher complexity in the bacterial composition compared to skin (Fig. 2, b) and oral mucosae (data not shown). Furthermore, in the skin lesional areas of pemphigoid patients *Staphylococcus* and *Corynebacterium* species outnumbered the others (Fig. 2, b). Conversely, we observed that pemphigus skin samples (Fig. 2, b) were characterized by an overall increased diversity of the bacterial species. To better understand the microbial composition results, we evaluated the alpha-diversity by mean of the Shannon index. This offers an estimation of the complexity and the overall richness of the taxa within the assayed populations. In our cohort, the abovementioned index was calculated for stool and skin samples in all patients. To note, we excluded the oral swabs data from this statistical analysis, due to the availability of only one sample belonging to the BP group. Finally, we checked potential differences in the alpha diversity. The Mann-Whitney test revealed a slight statistical significance ($p = .049$) only for the cutaneous alpha diversity between pemphigus and pemphigoid groups.

4. Discussion

In addition to the common skin barrier disfunctions and to the well-known immunologic disturbances, strong evidence supports the alteration of microbial composition as a common cause of skin disorders development and worsening. The healthy CM consists of both commensal and pathogenic bacteria, it affects the skin barrier, and it could be involved in antigen-driven disorders characterized by damage of the skin like psoriasis, eczema and, as recently demonstrated, in autoimmune blistering disorders (Ellebrecht et al., 2016; Miodovnik et al., 2017; De Benedetto et al., 2012).

Beforehand, this research field was affected by the intrinsic limitations of conventional bacteria culture-dependent methods. Culture-independent methods, such as Fluorescence In Situ Hybridization (FISH) (Gersdorf et al., 1993), Restriction Fragment Length Polymorphism (RFLP)(Takeshita et al., 2009), Pulse-Field Gel Electrophoresis (PFGE) (Capoluongo et al., 2000) and microarrays (Lif Holgerson et al., 2011), have gradually expanded the list of candidate strain pathogens associated with diseases. Among the latter techniques, full-length sequences generated by Sanger sequencing of 16S rRNA clone libraries is considered the gold standard for the phylogenetic evaluation, even if the largest studies analysed up to thousands of sequences for each samples (Allali et al., 2017). In the last decade, NGS-based technologies, together with new bioinformatics strategies, have boosted their performance to detect and characterise skin and gut bacteria (Song et al., 2018) in comparison with older PCR based methods (Pietravalle et al., 1999).

In this pilot study, we evaluated the microbial composition of lesional skin, oral mucosae and gut of patients affected by the two most important autoimmune bullous diseases, PV and BP.

The analysis of intestinal microbiota showed a relative abundance of Firmicutes and Bacteroidetes both in PV and BP patients, with an underrepresentation of Proteobacteria. The microbial intestinal composition at phyla level of healthy subjects is largely characterized by Firmicutes and Bacteroidetes, while Fusobacteria and Proteobacteria are only partially present. Our results are in line with literature data, showing a normal composition of intestinal microbiota. Furthermore, we have not highlighted significant differences in the relative abundances of these phyla between the two groups of patients.

By evaluating the lesional skin microbial composition, Firmicutes resulted the most abundant phylum in both PV and BP groups, as also described in perilesional sites of BP patients by Miodovnik et al. (Miodovnik et al., 2017). The results obtained from the skin samples also underlined the *Staphylococcus* genus as the most represented in both patients' groups; thus providing additional evidence of the role of

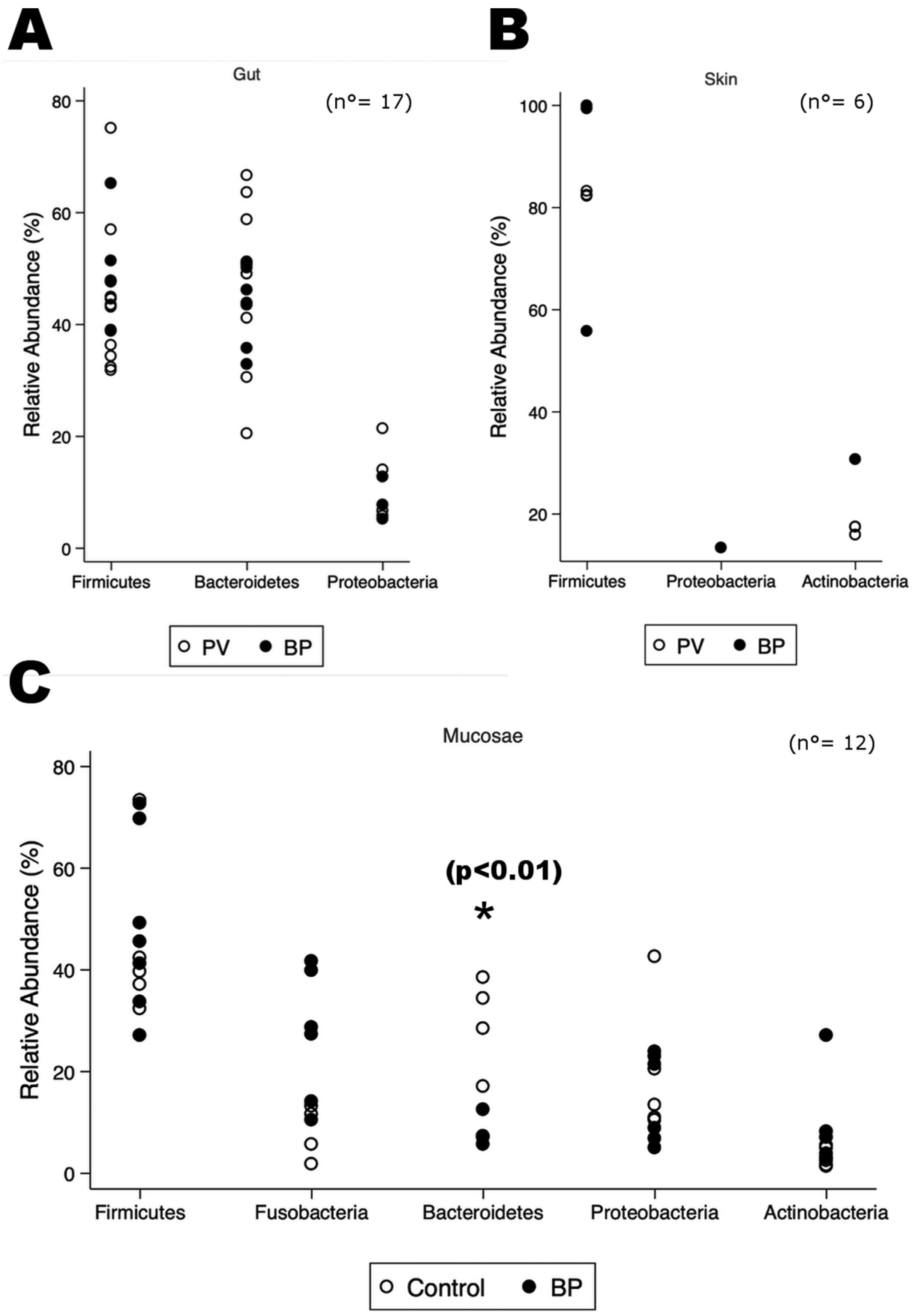
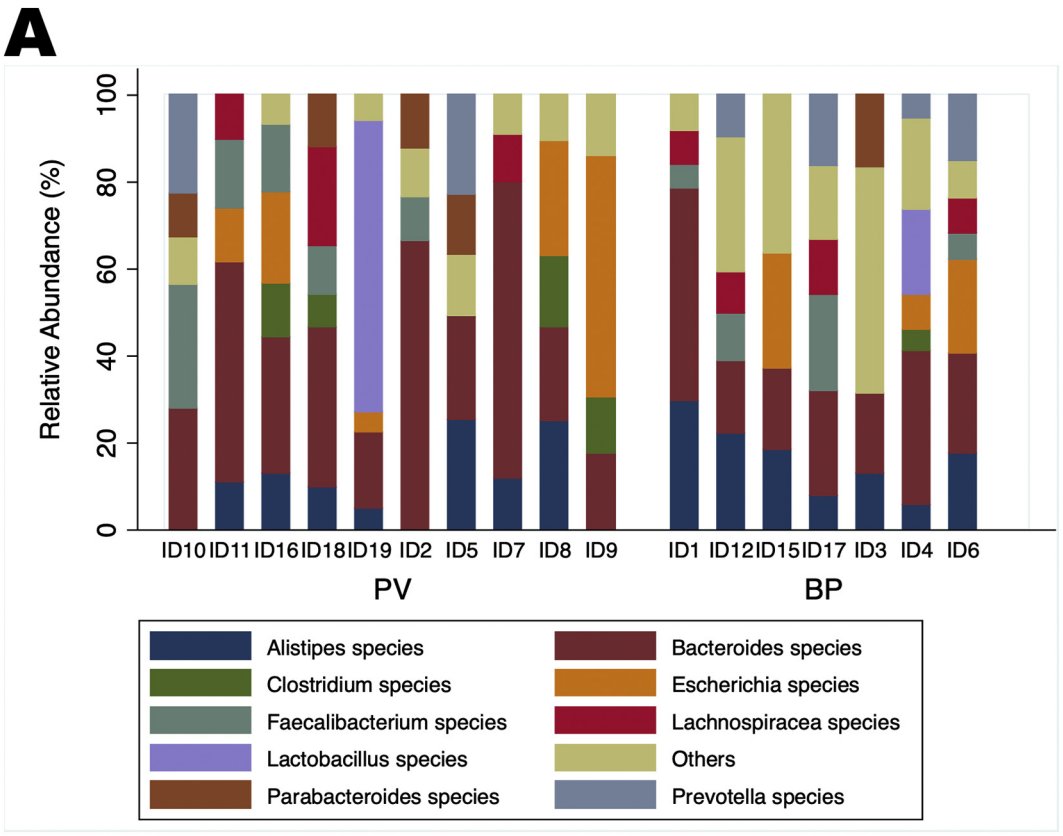


Fig. 1. Relative abundances (%) of the phyla (> 5%) in pemphigus, pemphigoid and control groups in gut (a), skin (b) and mucosae (c) site. In panel (c), the statistical significance of Mann-Whitney test is reported for the Bacteriodetes phylum.



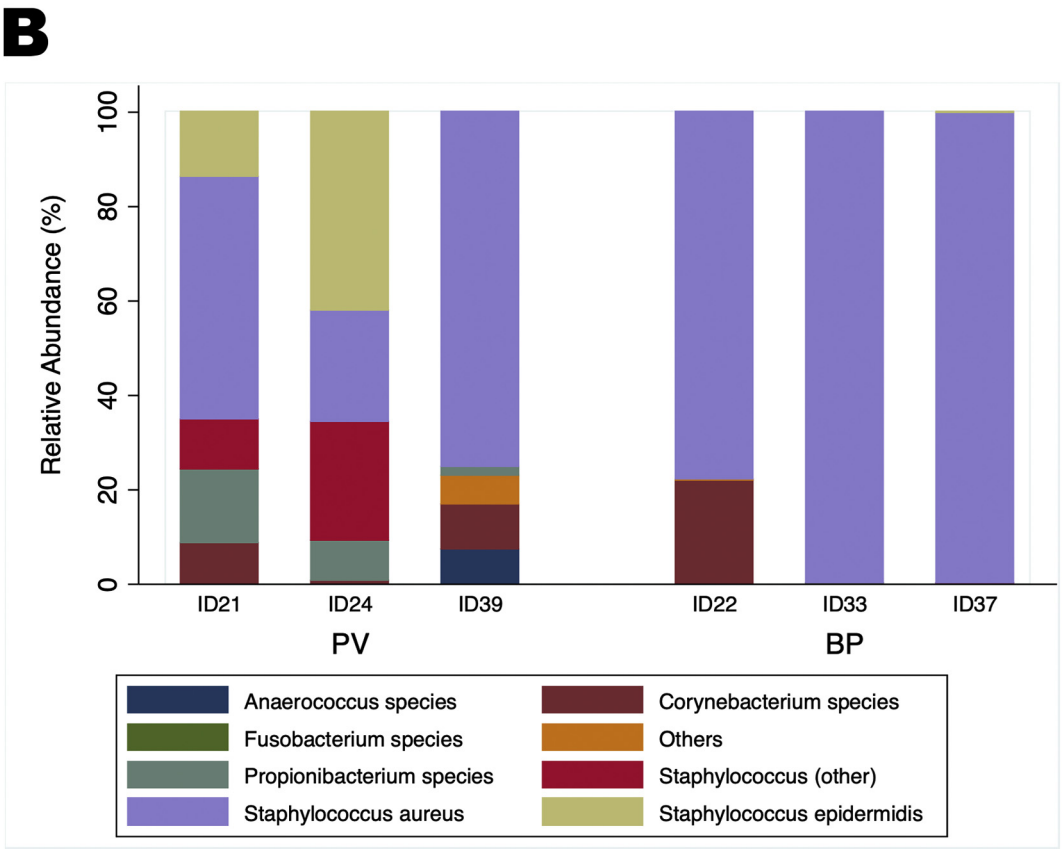


Fig. 2. Relative abundances (%) of the 10 most common species in both pemphigus and pemphigoid patients in gut (a) and skin (b) sampling site.

this phylum in the mechanisms triggering pathogenesis of the bullous diseases. The *Staphylococcus* genus, especially *S. epidermidis*, is a dominant group in healthy CM. Although *S. epidermidis* can play as an opportunistic pathogen in the context of immunosuppression, it predominantly acts as a mutualistic bacterium modulating the local immune reactions (Chen et al., 2018b). One of the main pathogens of the skin is the *S. aureus*, which is also involved in the pathogenesis of chronic disease like Atopic Dermatitis (AD)(Capoluongo et al., 2001; Yamazaki et al., 2017) and Systemic Lupus Erythematosus (LES) with skin involvement (Chen et al., 2018b). Particularly, a strong correlation was observed between AD severity and *S. aureus* relative abundances (Byrd et al., 2017). Finally, *Staphylococcus* is also reported as the prevalent genus in epidermolysis bullosa (Srinivas et al., 2013). Despite the relatively small number of subjects, data obtained on our patients' cohort are aligned with those published elsewhere, when phyla and genus levels are considered. However, in BP patients we found a prevalence of *S. aureus* while Miodovnik et al. reported a high relative abundance of the *S. epidermidis* (Miodovnik et al., 2017*)*. Furthermore, Actinobacteria phylum was found in all pemphigus skin swab samples and only in one BP subject. Noteworthy, Miodovnik et al.*(*Miodovnik et al., 2017*)* reported that Proteobacteria represent the most abundant phylum in the skin of healthy control and in the non-lesional sites from BP patients. Similarly to this result, Proteobacteria phylum was identified above our cut-off (> 5% of prevalence) in only one BP patient, confirming its lower pathogenicity as compared to Firmicutes. Due to the low number of patients with skin lesions enrolled in this pilot study, we were not able to compare cutaneous microbiome with healthy control subjects. Consequently, we planned to increase the number of subjects to perform more reliable, consistent analysis on our skin dataset.

Our results obtained from the oral mucosae samples of PV patients showed a qualitative higher diversity in bacterial phyla composition compared to the those from gut and skin swab's (Schmidt et al., 2014). Generally, Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria and Fusobacteria are reported in the healthy oral microbiome and, it is characterized by a relatively small number of bacterial phyla (Aas et al., 2005; Schmidt et al., 2014; Keijser et al., 2008; Zaura et al., 2009; Nasidze et al., 2009a; Nasidze et al., 2009b; Bik et al., 2010; Stahringer et al., 2012; Pushalkar et al., 2011; Pushalkar et al., 2012). Regarding the oral cavity of our patients, we detected Firmicutes phylum at higher relative abundance, followed by Fusobacteria, Proteobacteria, Actinobacteria, and Bacteroidetes. Notably, alterations of oral mucosae in BP are less frequent than those with pemphigus disease. In our cohort, oral mucosae samples were available for patients with pemphigus only, thus we compared PV OM with a selected reference control group (Schmidt et al., 2014). In this analysis, we found a statistically significant decrease of Bacteroidetes phylum in the oral mucosa of pemphigus patients compared to healthy control levels. We highlighted the typical oral *fetor* as a feature in pemphigus patients. This condition is not reported for the other bullous diseases. Volatile sulfur compounds and malodorous fatty acids produced from the decomposition of sulfur-containing amino acids, peptides and proteins by oral bacteria are considered as a direct cause of intra-oral halitosis (Murata et al., 2002). Moreover, differences within the microbial diversity between patients with intra-oral halitosis and healthy controls were previously reported (Kazor et al., 2003). We can speculate that the imbalance in microbial composition observed in PV patients may be involved in this typical clinical sign. Likewise, OM could trigger bullous diseases, mainly PV, whilst beginning in the oral cavity. Further efforts are required to understand the potential role of OM on this typical feature in PV patients.

To our knowledge, this is the first preliminary study evaluating the microbial composition at the three different sites, oral cavity, skin and gut, in two out most important types of bullous diseases. In literature, only two recent papers focused on the CM in bullous diseases (Ellebrecht et al., 2016; Miodovnik et al., 2017). Ellebrecht et al. *(*Ellebrecht et al., 2016*)* studied the CM by analysing the development of clinical symptoms in EBA-susceptible SJL/J mice. They found that in genetically identical mice, kept under highly standardized conditions, an increased richness and diversity of the CM before immunization led to a reduction of the blister formation. In fact, despite the presence of autoantibodies, 20% of SJL/J mice did not develop skin blisters. Also, Miodovnik et al.*(*Miodovnik et al., 2017*)* compared the CM of twelve BP patients to a similar number of matched controls at numerous distinct sites. They found that beta diversity, representing the overlap or dissimilarity between multiple populations, was significantly different between patients and controls in perilesional sites ($p < .001$) and within patients between perilesional and non-lesional sites ($p < .001$). Furthermore, they found that differences were much more prominent between patients and controls than within patients, in terms of phyla abundance.

To note, the low number of patients enrolled can represent the main limit of the present study. Nevertheless, it must be pointed out that both PV and BP are very rare conditions. In this pilot study, we decided to enroll only patients who were not receiving medications affecting microbiota analysis. In order to increase patient cohort and confirm these preliminary findings, we have planned a more extensive study.

5. Conclusion

A deeper knowledge of microbiome composition and microbe-host interactions will contribute to clarify the mechanisms of rare skin disorders. To date, there is still a lack of literature data regarding the impact of bacterial population in the development of bullous disease. Overall, our NGS approach provided a preliminary characterization of the microbiota in pemphigus and bullous pemphigoid, as evaluated in different sampling site (stool, lesional skin and oral mucosa). Firmicutes resulted the most represented phylum in the oral cavity and skin lesions in both PV and BP. A high relative abundance of *Staphylococcus* genus was found in PV (*S. aureus* and *S. epidermidis*) and BP (*S. aureus*) patients. Moreover, when compared to healthy controls, we found a significant decrease in *Bacteroidetes* phylum abundance in PV oral mucosae. Overall, these results suggest a possible role of Firmicutes phylum in the pathogenesis of the bullous diseases, as also demonstrated in other dermatological conditions. In particular, within phylum of Firmicutes, the *S. aureus* was the prevalent represented species in our samples. In this regard, we underline as it has been already reported (https://onlinelibrary.wiley.com/doi/pdf/10.1002/psb.392) that the colonization or the secondary infection by *S. aureus* can be present in these patients. We cannot establish who drove who, since itching and scratching could have also contributed to the raising of *S aureus* load within skin wounds.

The imbalance related to the significant reduction of Bacteroidetes in PV oral cavity may be associated to the typical *fetor* observed in these patients. Finally, as previously published, GM composition does not appear to play a particular effect on PVs and BP conditions.

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Declaration of Competing Interest

The authors declare no conflict of interest.

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