

# Characterisation of crevicular fluid microbiota in primary Sjögren's syndrome

G.A. Martínez-Nava<sup>1,2</sup>, A. López-Reyes<sup>1,2</sup>, C. Hernández-Hernández<sup>3</sup>,  
V. Ruiz-González<sup>3</sup>, A. Llorente-Chávez<sup>4</sup>, V. Saavedra-González<sup>4</sup>,  
L. Llorente<sup>4</sup>, G. Hernández-Molina<sup>4</sup>

<sup>1</sup>Laboratorio de Gerociencias, Instituto Nacional de Rehabilitación Luis Guillermo Ibarra Ibarra, Mexico City; <sup>2</sup>Centro de Investigación Sobre el Envejecimiento, Mexico City;

<sup>3</sup>Servicio Dental, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Mexico City;

<sup>4</sup>Departamento de Inmunología y Reumatología, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Mexico City, Mexico.

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## Abstract

### Objective

To describe the taxonomy of the microbiota in crevicular fluid of primary Sjögren's syndrome (pSS) patients, and evaluate its association with clinical/serological variables, and oral quality of life.

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### Methods

Observational study that included 48 pSS without diabetes mellitus, no active neoplasia, no antibiotic use in the previous two weeks, and no current active infection. We registered demographics, oral/ocular sicca symptoms, parotid enlargement and anti-Ro/La serology. We assessed the non-stimulated whole salivary flow (NSWSF), the EULAR Sjögren's Syndrome Patient Reported Index (ESSPRI), and the Xerostomia-related Quality of Life Scale (XeQoLS). Two periodontists determined the presence of periodontal disease and collected crevicular fluid from 6 teeth using filter paper. Samples were frozen at -86°C until processing. We included 17 sex- and age-matched control subjects. Bacterial DNA was extracted from the crevicular fluid sample using a commercial kit. 16SrRNA V3-V4 region was sequenced using reversible adaptor technology. Sequences were pre-processed and analysed using QIIME2 and phyloseq software programs. Functionality profiles were predicted using the Tax4Fun2 package.

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### Results

PSS patients had more bacteria of the genera *Prevotella*, *Streptococcus*, *Veillonella*, *Fusobacterium*, and *Leptotrichia* and fewer bacteria of the genus *Selenomonas* than controls. The pSS microbiota contained more genes encoding accessory secretory proteins. Microbiota also differed between patients with anti-Ro/La status, parotid gland enlargement, and periodontal disease severity, but did not correlate with NSWSF and XeQoLS.

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### Conclusion

The crevicular fluid microbiota of pSS patients and controls differed significantly, even in SSP patients depending on their serology, parotid gland enlargement, and periodontal disease status.

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### Key words

Sjögren's syndrome, microbiota, crevicular fluid

Gabriela Angélica Martínez-Nava, PhD  
 Alberto López-Reyes, PhD  
 Carlos Hernández-Hernández, MD  
 Vanessa Ruiz-González, MD  
 Amaya Llorente-Chávez, MD  
 Vanessa Saavedra-González, MD  
 Luis Llorente, MD  
 Gabriela Hernández-Molina, MS  
 Please address correspondence to:  
 Gabriela Hernández-Molina  
 Department of Immunology and  
 Rheumatology,  
 Instituto Nacional de Ciencias Médicas  
 y Nutrición Salvador Zubirán,  
 Vasco de Quiroga 15, Col. Belisario  
 Domínguez Sección XVI,  
 CP 14080 Mexico City, Mexico.  
 E-mail: gabyhm@yahoo.com  
 ORCID iD: 0000-0001-7958-0391

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## Introduction

Primary Sjögren's syndrome (pSS) is a chronic autoimmune condition characterised by inflammation of the exocrine glands, primarily the salivary and lacrimal glands. Although not fully understood, its aetiopathogenesis encompasses the interaction of environmental, genetic, and immunological variables leading to changes in the glandular epithelium (1). Recently, it has been shown that microbiota also plays a role in the pathogenesis of several autoimmune diseases, including PSS. Therefore, some processes that link the microbiome and PSS may include molecular mimicry, modifications in metabolites like short-term acids (implicates, for instance, in the Treg/Th17 balance), loss of epithelial tolerance, and mucosal barrier (2).

Currently, there are various reports in pSS where the intestinal microbiota has been investigated in stool samples, other studies have analysed conjunctival exudates, and the majority have studied the oral cavity, either through non-stimulated saliva samples or oral rinses or swabs. In this regard, *Proteobacteria* (3, 4), *Synergistetes*, *Spirochaetes* (4, 5), *Proteobacteria* (6-7), *Firmicutes* (8), *Fusobacteria* (4), have been found to be less prevalent in the oral cavity of patients with PSS, whereas other studies have found an increase in *Firmicutes* (4, 5, 8), the F/B ratio (9) *Bacteroidetes* (7), *Actinobacteria* and *Proteobacteria* (8). However, it remains unclear whether the oral microbiota can affect oral symptoms, decreased salivation, oral quality of life, and systemic activity.

Gingival crevicular fluid is an inflammatory exudate derived from the periodontal pockets. Its volume is normally minimal and rises with an inflammatory response. It is made up of a combination of bacteria, neutrophils, and deteriorating tissue. It is gathered using a non-intrusive and straightforward technique (10). Only one study has so far examined the microbiome in pSS gingival crevicular fluid. That study showed that there were equal numbers of bacteria in the periodontal sites of patients with pSS and healthy controls, and that periodontal therapy had no effect on the bacterial population. However, only a

small number of bacteria were examined, including *P. gingivalis*, *A. actinomycetemcomitans*, *T. forsythia*, and *T. denticola* (11). It is also compelling to note that several variables, such as age and the presence of periodontitis and caries, which are common conditions in pSS, can affect the results.

The purpose of this study was to describe the taxonomic composition of the oral microbiota in the crevicular fluid of patients with pSS compared to healthy controls and to evaluate its correlation with oral symptoms, non-stimulated whole salivary flow, the presence of anti-Ro/SSA and anti-La/SSB, periodontal disease, and oral quality of life.

## Materials and methods

### Patients

This was an observational study carried out in a tertiary care centre in Mexico City. We included 48 consecutive patients who fulfilled the American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) classification criteria (12) for pSS. We excluded patients with other concomitant connective tissue disease, diabetes mellitus, active neoplasia, use of antibiotics in the 2 weeks prior to the study, or who had an active infection at the time of the study.

A single rheumatologist interviewed the patients and registered the following variables: demographics, disease duration, oral and ocular sicca symptoms, parotid gland enlargement, and current treatment (steroids, immunosuppressants, antimalarials). We also registered anti-Ro/SSA and anti-La/SSB antibodies from the medical chart. We scored the ESSDAI activity index (13) at the time of the visit.

We also evaluated the non-stimulated whole salivary flow (NSWSF) in a standard fashion (14). Patients rated the EULAR Sjögren's Syndrome Patient Reported Index (ESSPRI) (13) as well as the oral quality of life using the Xerostomia-related Quality of Life Scale (XeQoLS) (15).

### Dental evaluation and sampling of crevicular fluid

All patients were evaluated by two expert periodontists in a standardised

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manner. We evaluated six sites per tooth regarding probing depth, clinical attachment levels, and bleeding on probing, scored the Simplified Oral Hygiene Index (16), and assessed the presence of periodontal disease. We classified the periodontal disease as generalised (>30% of the sites evaluated with loss of clinical attachment) or localised ( $\leq 30\%$ ); as well as mild, moderate, and severe. Likewise, the number of missing, filled, and decayed teeth were evaluated. Crevicular fluid was collected from 6 teeth with the greatest periodontal pocket depth using filter paper (sterile paper points). Samples contaminated with blood were discarded. Samples were immediately frozen at  $-86^{\circ}\text{C}$  until processing.

We also included 17 control subjects attending a regular appointment to the Dental department matched by sex and age ( $\pm 5$  years). We excluded controls with a diagnosis of autoimmune disease, diabetes mellitus, active neoplasia, periodontal disease, use of antibiotics within 2 weeks prior to the study, or who had an active infection at the time of the study, but they might have other comorbidities.

### Microbiota sequencing

The bacterial DNA was extracted from the crevicular fluid sample using a commercial kit (ZymoBIOMICS DNA Miniprep Kit, Zymo Research, USA). Bacterial DNA was quantified by spectrometry and fluorometry, and only those samples with a minimum A260/280 ratio of 1.9 were used for amplification of the V3-V4 variant region of the 16S rRNA gene. The generated amplicons were column purified and sequenced with reversible terminator technology on the MiSeq (Illumina). Using dual indices, sequencing was performed with the MiSeq Reagent kit v3 (600 cycles) that included 20% of the PhiX sequencing control in each run.

### Bioinformatics and statistical analysis

We performed the bioinformatics analysis using the DADA2 algorithm on the QIIME2 platform for sequence quality control (denoising) and to group sequences into amplicon sequence variants (ASVs). Using the filtered ASVs,

**Table I.** Clinical and serological features of the patients.

Variable	Primary Sjögren (n=48)
Ocular symptoms, n (%)	45 (93.7)
Oral symptoms, n (%)	43 (89.5)
Parotid gland enlargement, n (%)	16 (33.3)
Anti-Ro/SSA positivity, n (%)	42 (87.5)
Anti-La/SSB positivity, n (%)	26 (45.8)
Rheumatoid factor, n (%)	23/40 (57.5)
High globulins, n (%)	16/42 (38)
Low C3, n (%)	3/41 (7.3)
Low C4, n (%)	9/41 (21.9)
Focal lymphocytic sialadenitis, n (%)	27/37 (72.9)
Tobacco use, n (%)	15 (31.2)
Median NSWSF <sup>§</sup> in ml/15 min (IQR)	1 ml (0.5-2)
Median ESSPRI <sup>†</sup> , (IQR)	5.9 (4.7-8)
Median XEQoLS <sup>‡</sup> , (IQR)	30 (21.5-39.7)
Median ESSDAI <sup>§</sup> , (IQR)	0 (0-2)
Antimalarials, n (%)	21 (43.7)
Prednisone, n (%)	5 (10.4)
Immunosuppressors <sup>¶</sup> , n (%)	11 (22.9)
Biological treatment, n (%)	0 (0)
Periodontal disease (%)	44 (97.78%)
Extension of periodontitis (%)	
Generalised	13 (29.55)
Located	31 (70.45)
Severity of periodontitis (%)	
Mild	21 (47.73)
Moderate / Severe	23 (52.28)
Median percentage bleeding on probing (IQR)	9.3 (3.7-26.3)
Median number of cavities, (IQR)	1 (0-5)
Median number of missing teeth (IQR)	2 (1-7)
Median number of filled teeth, (IQR)	4 (1-9)
Median healthy teeth, (IQR)	13.5 (11-19)
Simplified Oral Hygiene index, (IQR)	0.75 (0.66-1.27)

<sup>§</sup>NSWSF: non-stimulated whole salivary flow.

<sup>†</sup>ESSPRI: EULAR Sjögren's Syndrome Patient Reported Index.

<sup>‡</sup>XEQoLS: Xerostomia-related Quality of Life Scale.

<sup>§</sup>ESSDAI: EULAR Sjögren's syndrome disease activity.

<sup>¶</sup>Immunosuppressors: azathioprine (n=5), methotrexate (n=5), mycophenolate mofetil (n=1).

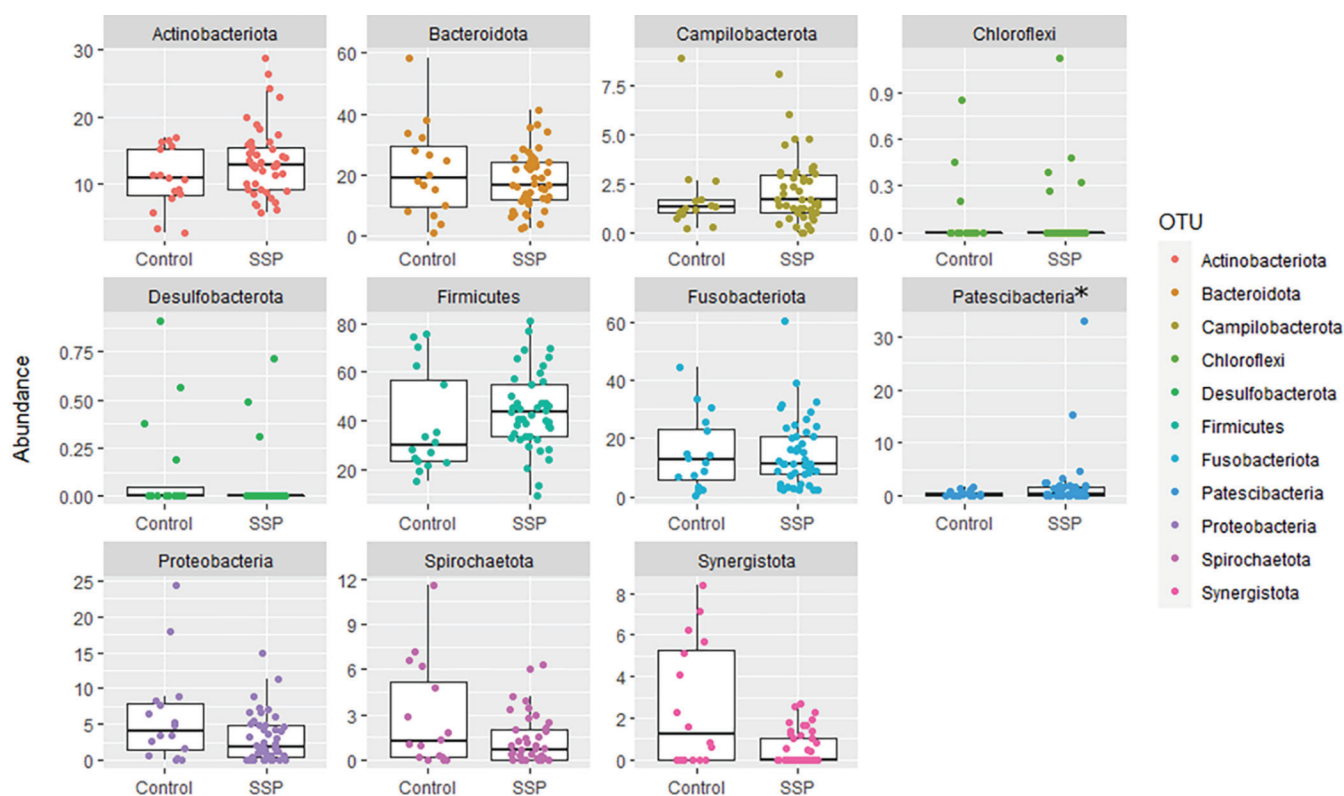
we assigned the taxonomic affiliations (phylotypes) based on a pre-trained Bayesian classifier to the region of interest with the curated database SILVA version 139.

For all statistical analysis significance level was set at  $\alpha=0.05$ . If specified, we performed multiple comparison correction methods, so *p*-values reported were false discovery rate (FDR) *p*-values.

We evaluated the  $\alpha$ -diversity by estimating different richness and diversity indices (the number of OTUS and the Chao1, Shannon, and Simpson indices) after the rarefaction of the samples. In the same way, we carried out a non-linear regression analysis of fixed effects to determine the radius of the consecutive frequencies observed between the study groups and, with this, to evaluate the alpha diversity without the possible bias introduced by rarefaction. We ana-

lysed the  $\beta$ -diversity of the crevicular fluid microbiota using weighted UNIFRAC scores, and principal coordinate analysis (PCoA) to assess the relationship between the  $\beta$ -diversity and the two study groups. We compared the scores obtained by each individual for the main coordinates using the Kolmogorov-Smirnov test.

To evaluate the differences in the relative abundances of the bacterial genera, three analytical approaches were carried out. The first was performed by comparing the relative abundances between pSS patients and controls using the rank sum parametric test. Subsequently, with the bacterial genera that showed a significant difference in this first approach, a linear discriminant analysis was performed using the LefSe tool, as well as a reduction estimation analysis for dispersions and



**Fig 1.** Identified bacteria phyla among studied groups.

\*Significant  $p$ -value ( $p=0.02$ ) obtained from rank sum test.

times of change with the DESeq2 algorithm. For these analyses we used Storey's method to calculate FDR and correct for multiple comparisons.

In order to assess the impact of the crevicular fluid microbiota profile and clinical covariates, the same diversity and relative abundance analyses were performed with the clinical variables that were relevant in the descriptive analysis of the study population. Likewise, for the discrete and continuous clinimetric variables, a correlation analysis was performed by calculating the Spearman correlation coefficient with the aforementioned variables and the relative abundances of the ASVs that had a significant difference between the study groups.

Finally, we evaluated the functional profile of the crevicular fluid microbiota predicted with Tax4Fun2 software (17). The relative abundance of bacterial proteins was compared between the two study groups using Welch's T test and correcting for multiple comparisons by calculating FDR using the Storey's method. This study conforms to STORMS statement.

#### Ethics statement

The study was approved by the ethics committee of the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubiran, and all the patients signed informed consent.

#### Results

Most of the patients (93.6%) were female, the mean age was  $52.4 \pm 11$  years, and the median disease duration was 7 years (IQR 4–11.2). The average age of the controls was 47.4 years, and their median unstimulated salivary flow was 4 ml (IQR 3–5). Table I shows the clinical and serological characteristics of patients. Regarding the dental evaluation, most of the patients with PSS presented periodontal disease (Table I).

#### Crevicular fluid microbiota

pSS patients showed a higher  $\alpha$ -diversity measured by the Shannon index than healthy subjects ( $p < 0.05$ ); however, no significant difference in  $\beta$ -diversity was observed between the study groups (data not shown).

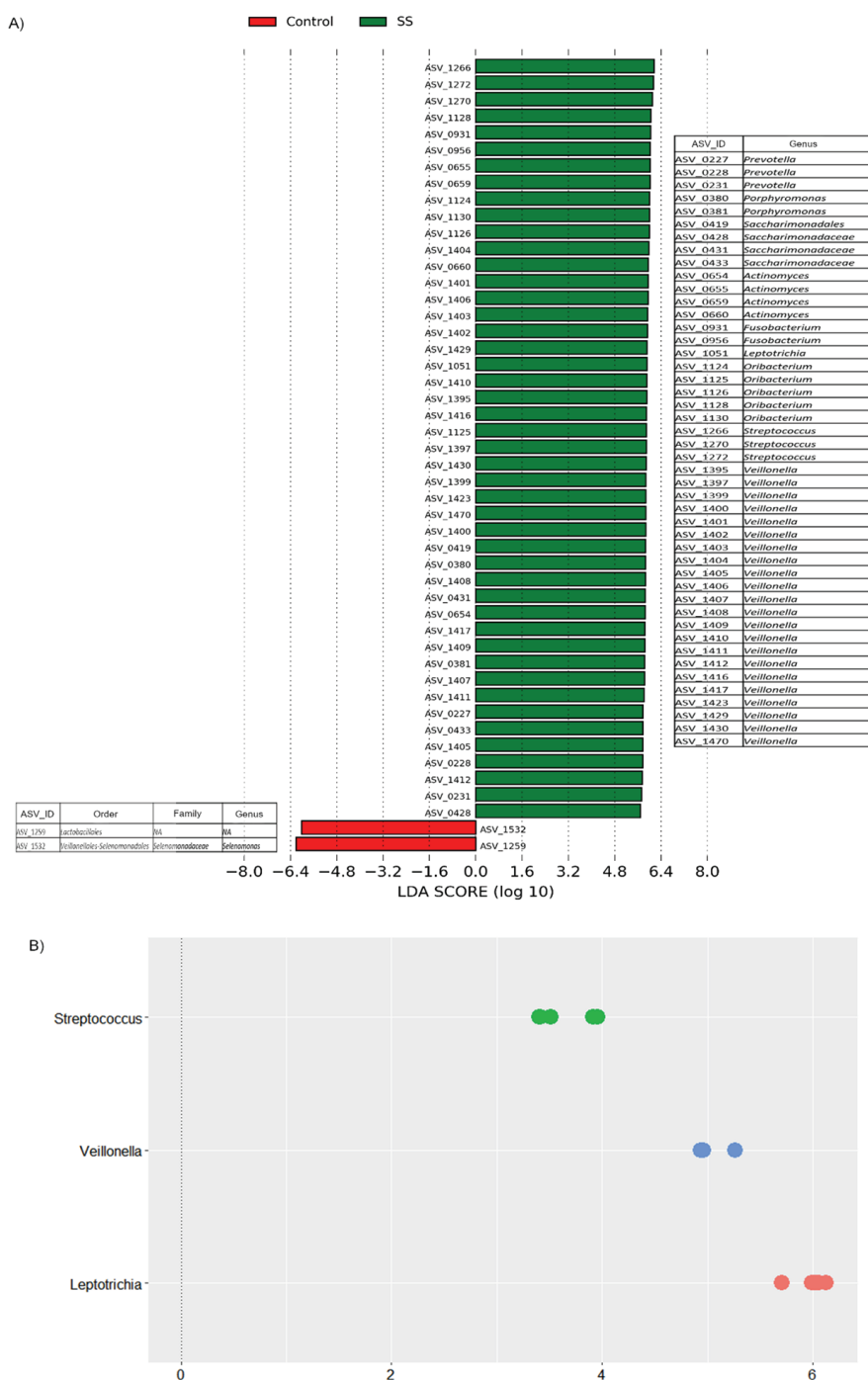
The microbiota of the crevicular fluid of patients with PSS was characterised by a

greater abundance of bacteria belonging to the *Patescibacteria* phylum ( $p=0.02$ ), however, no significant difference was observed in the relative abundance of the other detected phyla (Fig. 1).

At the genus level, patients with PSS had a greater abundance of bacteria of the genus *Prevotella* ( $p=0.04$ ), *Fusobacterium* ( $p=0.02$ ), *Streptococcus* ( $p=0.03$ ), *Veillonella* ( $p=0.003$ ), *Leptotrichia* ( $p=0.03$ ), *Oribacterium* ( $p=0.007$ ), *Actinomyces* ( $p=0.02$ ) and *Porphyromonas* ( $p=0.04$ ), as well as a fewer abundance of bacteria belonging to the genus *Selenomonas* ( $p=0.04$ ) and bacteria belonging to the order *Lactobacillales* ( $p=0.002$ ) compared to control subjects (Fig. 2).

When performing a multivariate regression model adjusting for age and salivary flow, it was found that 16 ASVs were significantly associated with the diagnosis of pSS, six of which belong to bacteria of the genus *Prevotella* ( $p=0.001$ ), two to the genus *Gamella* ( $p=0.002$ ), one to the genus *Veillonella* ( $p=0.05$ ), *Actinobacillus* ( $p=0.03$ ), *Corynebacterium* ( $p=0.03$ ), *Rothia* ( $p=0.02$ ), *Fretibacterium* ( $p=0.002$ ), *Oribacterium*





**Fig. 2.** (A) ASVs which best define each study group and (B) those that had a significant difference in the DeSeq2 analysis.

( $p=0.006$ ), *Selenomonas* ( $p=0.009$ ), and *Centipeda* ( $p=0.02$ ).

We performed a multivariate linear regression analysis to find out which bacterial genera were associated with a higher or lower salivary flow. We found that one ASV from the genus *Corynebacterium* and another belonging to the genus *Rothia* were significantly associated with a higher salivary

flow, adjusting for the presence of the disease ( $\beta=0.35$ ;  $p=0.036$  and  $\beta=0.49$ ,  $p=0.008$ , respectively). For the other bacterial genera, we did not find a significant association.

*Crevicular fluid microbiota and clinical and serological characteristics*  
When stratifying the patients by anti-Ro/SSA and anti-La/SSB antibody positiv-

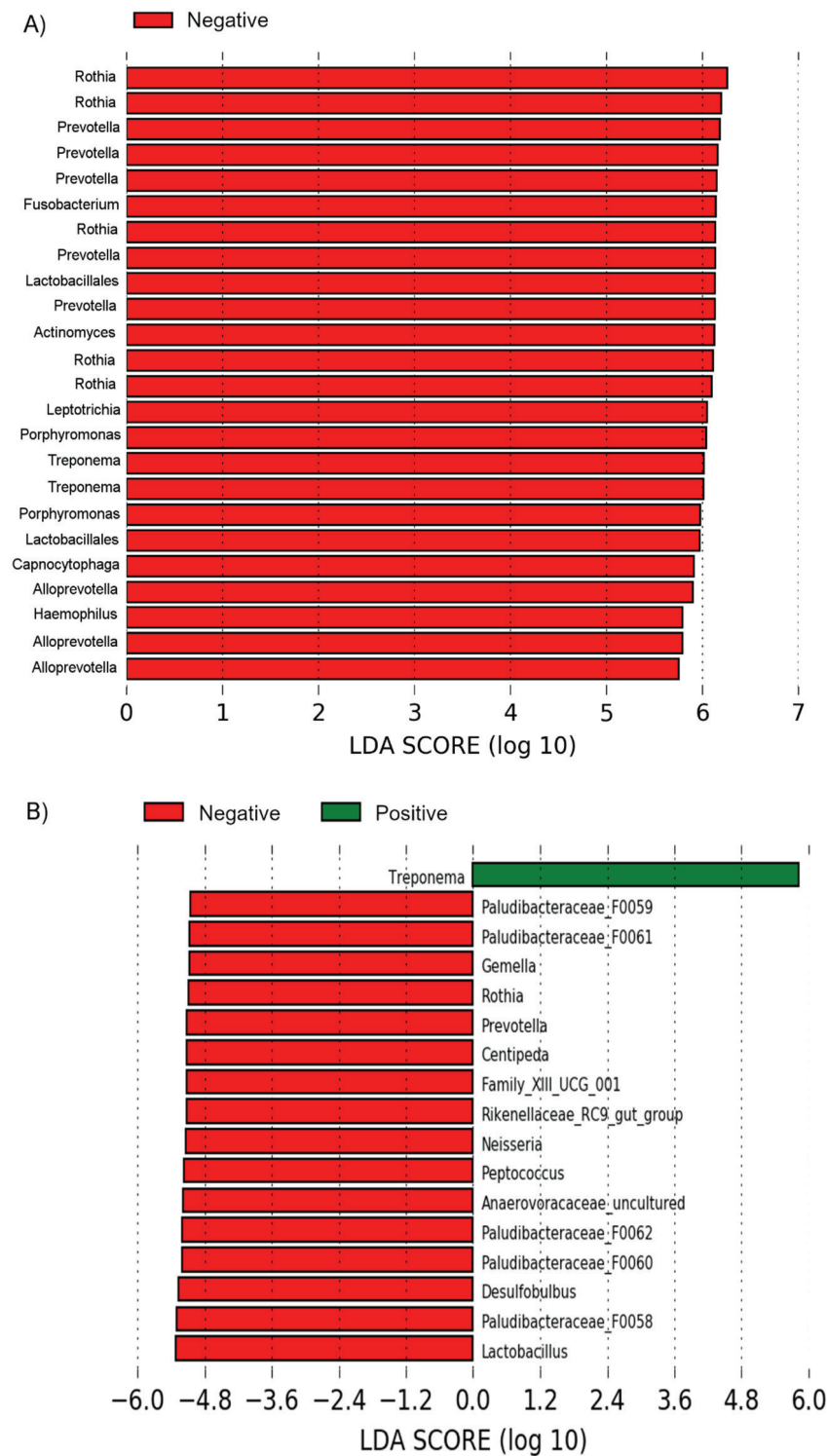
ity, as well as by the presence of parotid gland enlargement, we did not observe any significant difference in either  $\alpha$  diversity or  $\beta$  diversity (data not shown).

However, we were able to detect a significant difference in the abundance of bacteria of the *Campylobacterota* phylum among the patients with parotid gland enlargement, with a higher relative abundance than the patients without it ( $p=0.038$ ). In addition, 218 ASVs were identified with a different relative abundance in the crevicular fluid microbiota between positive and negative patients for each of the antibodies as well as for the presence of parotid gland enlargement.

When performing the linear discriminant analysis, we found that a greater abundance of 24 ASVs better characterised the microbiota of the crevicular fluid in negative anti-Ro/SSA or anti-La/SSB PSS patients compared to that of positive patients. Of these, five were *Rothia* bacteria ( $p=0.02$ ), five were *Prevotella* bacteria ( $p=0.02$ ), one was *Fusobacterium* bacteria ( $p=0.03$ ), two were *Actinomyces* bacteria ( $p=0.04$ ), two were *Porphyromonas* bacteria ( $p=0.04$ ), two were *Treponema* bacteria ( $p=0.02$ ), one was *Capnocytophaga* bacteria ( $p=0.03$ ), and two were *Alloprevotella* bacteria (Fig. 3A).

In the case of the parotid gland enlargement among PSS patients, the linear discriminant analysis showed that the microbiota of the crevicular fluid of these patients is characterised by a greater abundance of an ASV belonging to bacteria of the genus *Treponema* ( $p=0.02$ ). The microbiota of patients negative for parotid gland enlargement had a greater abundance of 16 ASVs belonging to the genera F0058 ( $p=0.001$ ), F0059 ( $p=0.01$ ), F0060 ( $p=0.004$ ), F0061 ( $p=0.03$ ) and F0062 ( $p=0.03$ ) from the *Plaudibacteraceae* family; *Gemella* ( $p=0.02$ ), *Rothia* ( $p=0.04$ ), *Prevotella* ( $p=0.02$ ), *Centipeda* ( $p=0.02$ ), *Neisseria* ( $p=0.04$ ), *Peptococcus* ( $p=0.008$ ), *Desulfobulbus* ( $p=0.02$ ) and *Lactobacillus* ( $p=0.02$ ), as well as from the *Rikenellaceae* ( $p=0.02$ ) and *Anaerovoracaceae* ( $p=0.04$ ) family (Fig. 3B).

Since almost all PSS patients had periodontal disease, we decided to evaluate the taxonomical profile among the



**Fig 3.** ASVs that best describe the crevicular fluid microbiota of SS patients and healthy controls: (A) SS patients negative to anti-Ro/SSA or anti-La/SSB and (B) SS patients with and without parotid enlargement.

severity of the periodontal disease. We did not observe any significant difference in  $\alpha$  or  $\beta$ -diversity; however, 128 ASVs had different relative abundance between those patients that had mild and those who presented moderate or

severe periodontal disease. With this significant ASVs we performed a linear discriminant analysis and found that patients with mild periodontal disease had higher relative abundance of an ASV belonging to *Lactobacillus* genus

( $p=0.03$ ) than patients having moderate or severe periodontal disease; and the latter had higher relative abundance of an ASV belonging to *Fretibacterium* genus than the former ( $p=0.04$ ) (Fig. 4). We performed a correlation analysis to assess whether the excess of any bacterial genera influenced the oral quality of life score; however, we did not observe any significant correlation.

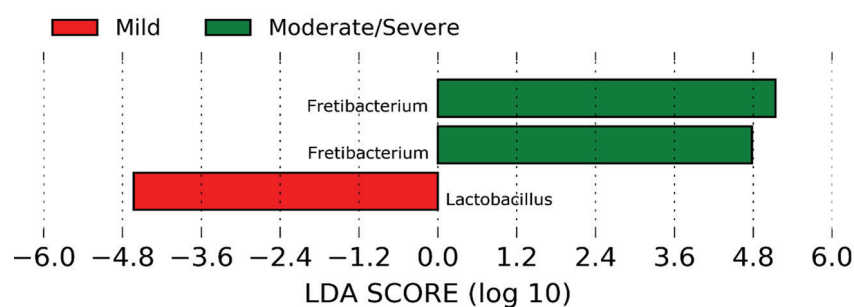
#### Functional profile prediction of the crevicular fluid microbiota

We analysed the different abundance of bacterial genes present in the crevicular fluid microbiota between the study groups. Patients exhibited a higher amount of bacterial genes encoding accessory proteins (Asp) 1-3 than controls ( $p=0.03$ ) (Fig. 5).

#### Discussion

Some recent studies have described changes in the distribution of the microbiota in patients with pSS. For example, at the intestinal level, a lower *Firmicutes/Bacteroides* (F/B) index has been described in comparison with controls, as well as a decrease in diversity in these patients (6, 19). On the other hand, studies of the oral microbiota in PSS have primarily focused on saliva or oral swabs, and an increase in the *Firmicutes*, *Bacteroides*, *Actinobacteria*, and *Proteobacteria* phyla has been described (4, 5, 7-9). It is important to highlight that the different structures of the oral cavity have different bacterial flora. Thus, a study in which nine sites of the oral cavity were sampled, different bacterial flora were found in each. *Streptococcus* and *Gemella* predominated in the gingival plaque and oral epithelium whereas *Prevotella*, *Porphyromonas*, and *Firmicutes* predominated in the tonsils (20).

Recently, a study evaluating anal swab samples, saliva samples, and vaginal swab samples found that the abundance of microbiota from these three sites differed in pSS patients vs. healthy controls. Lower abundance of *Proteobacteria* and *Spirochaetota* and higher abundance of *Firmicutes*, *Bacteroidota* and *Cyanobacteria* were documented at the oral level. At the vaginal level, only lower abundance of *Proteobacteria*



**Fig 4.** Bacteria genus ASVs whose relative abundance best describe the crevicular fluid microbiota of SS patients with mild and moderate or severe periodontal disease.

was found in pSS, and at the intestinal level, patients had lower abundance of *Actinobacteriota* and higher abundance of *Bacteroidota*, and a lower R/B ratio. When compared with a group of patients with sicca symptoms without PSS, the intestinal and vaginal microbiota was similar in patients with pSS, and the oral microbiota differed slightly, within a greater abundance of the F/P ratio and four genders in pSS (21).

Crevicular fluid analysis provides the opportunity to obtain specific information about the gingival area, a structure frequently affected in pSS patients. Although this information can be considered site-specific, we took samples from six different teeth or the four quadrants to obtain a general picture. We documented a greater presence of the phyla *Patescibacteria*, *Prevotella*, *Streptococcus*, *Veillonella*, *Fusobacterium*, and *Leptotrichia* and a lower abundance of bacteria belonging to the genus *Selenomonas*. Our findings contrast with the only study that examined crevicular fluid and found no differences in the microbiota of patients with pSS (with and without periodontitis) and healthy controls (with and without periodontitis). However, it only analysed the presence of *P. gingivalis*, *A. actinomycetemcomi-*

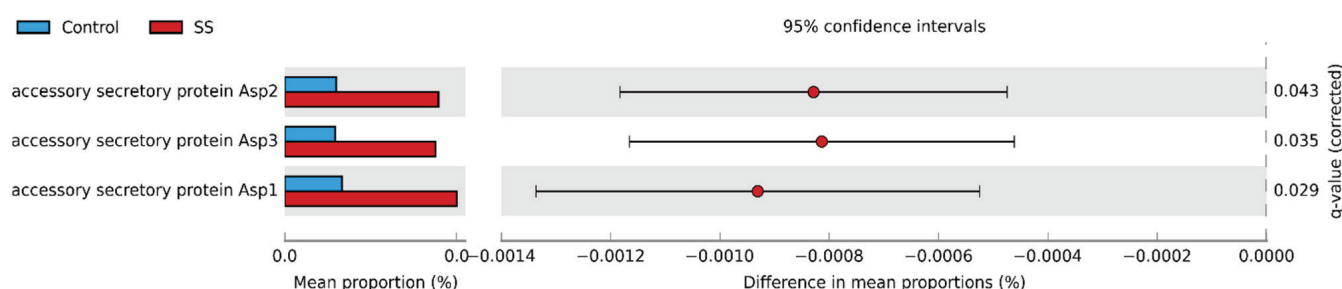
*tans*, *T. forsythia*, and *T. denticola*. Furthermore, periodontal treatment in that study did not reduce bacterial counts in patients with pSS (11). In another study of pSS patients, in which the supragingival and subgingival biofilm was examined with curettes, a greater presence of *Veillonella parvula*, *Fusobacterium nucleatum*, and *Propionibacterium* was observed in the supragingival samples, whereas a greater amount of *V. parvula* and a lower presence of *T. forsythia* and *Porphyromonas gingivalis* in the subgingival samples (21).

It has been postulated that the difference in oral microbiota may be due to hyposalivation. A study that evaluated oral washes of pSS patients found that the relative abundance of *Haemophilus*, *Neisseria*, and *Lactobacillus* correlated with salivary flow in patients, controls, and sicca patients without pSS (22). Another study also found no difference in salivary microbiota between patients with PSS and non-SS sicca controls (23). Siddiqui *et al.* conducted a study involving PSS patients with normal salivation (>1.5 ml in 15 min) and healthy controls and examined the microbiota in saliva. They found differences in six phyla: *Firmicutes*, *Bacteroidetes*, *Proteobacteria*,

*Actinobacteria*, and *Fusobacteria*; and in patients with SS, the species *Veillonella parvula*, *Veillonella atypica*, *Prevotella melaninogenica*, and *Prevotella histicola* were more conspicuous. The authors postulated that dysbiosis of the salivary microbiota can occur independently of hyposalivation (5).

This concept was also supported by Rus-then *et al.*, who found differences in the microbiota between the PSS vs. healthy, sicca vs. healthy, and PSS vs. sicca groups. A greater presence of *Granulicatella adiacens* was detected in the sicca group. But when only the patients with hyposalivation from the pSS vs sicca group were analysed, differences in *Actinomyces odontolyticus*, *Campylobacter concisus*, *Actinomyces lingnae*, *Lachnoanaerobaculum orale*, *Megaphaera micronuciformis*, *Corynebacterium asaccharolyticum*, *Capnocytophaga leadbetteri*, *Prevotella nanceiensis*, *Granulicatella adiacens*, and *Prevotella nanceiensis* were also found (24). Our results regarding bacterial genera between patients and controls were adjusted for salivary flow and remained significant. However, the genera *Corynebacterium* and *Rothia* were associated with higher salivary flow.

Regarding the anti-Ro/SSA and anti-La/SSB antibodies status, a study of 23 patients with pSS found differences in the salivary microbiota between the positive and negative anti-Ro/SSA groups. Patients seropositive for anti-Ro/SSA had higher abundance of the *Firmicutes* phylum and lower abundance of the *Actinobacteria* and *Proteobacteria* phyla. Lower phyla diversity and abundance was also observed in the anti-Ro/SSA positive group (25). In crevicular fluid, we also observed differences in the microbiota of seropositive patients



**Fig. 5.** Predicted bacterial coding genes in the crevicular fluid microbiota that had significant different abundance between SS patients and controls.



for either anti-Ro/SSA or anti-La/SSB. This phenomenon could be explained by molecular mimicry, genetic factors, mainly HLA, or by autoimmunity itself. In addition, we evaluated the diversity of the microbiota regarding the presence of parotid gland enlargement, finding some differences. In contrast, a previous study of saliva samples did not (25). However, our finding is relevant, as parotid glands are the main site of haematological neoplasia in pSS, so the dysbiosis associated with parotid gland enlargement could cause chronic damage and a higher risk of lymphoma in this organ (26).

In the context of periodontitis, bacteria of the genus *Fretibacterium* were found in higher abundance in the salivary microbiota of patients with periodontitis than in healthy participants (27, 28). Herein, we found higher abundance of this genus in pSS patients who had more severe periodontal disease compared to those with only mild disease. We also investigated whether the microbiota could influence oral quality of life, finding no association.

Finally, regarding the predicted functional profile, we observed a higher number of bacterial genes encoding three Asp in the microbiota of pSS patients than in controls. Many pathogenic bacteria use specialised protein secretion systems during infection, and it is quite possible that humans develop antibodies against the proteins involved in the secretory pathway of bacteria (29-30). Bacteria have evolved mechanisms to evade this immune response, thus it is not surprising that many secretory proteins in bacteria share similarity with human secretory proteins (31).

It is important to mention that the results in microbiota studies may be influenced by sample location, age, comorbidities, and medication use. For example, the presence of *Lactobacillus* and *Streptococcus* in patients with pSS might be altered by prednisone intake (3), and hydroxychloroquine can partially modify microbiota dysbiosis (20). Another important factor that could influence the identified bacteria profile is the selection of the hypervariable region of the sequenced 16sRNA as there is no consensus. Herein, we focused on

the V3-V4 region as done in most of the previous studies (4, 7, 9, 24, 25, 28, 29). Moreover, the type of control may also hamper the interpretation of our results (32). For instance, we did not include a subgroup of patients with only sicca symptoms. Nevertheless, there are some studies that have reported no differences in the salivary microbiota of patients with pSS and sicca patients (23). In addition, we also did not include a control group with periodontitis, however we were still able to explore the severity of periodontal disease among the pSS group. Finally, we did not consider other factors that might influence the microbiota such as smoking, drinking, and other oral health status variables besides the salivary flow. In conclusion, this is the first study to show a comprehensive analysis of the microbiota in the crevicular fluid of patients with pSS. We observed significant differences when comparing the composition versus healthy subjects. Differences were also found between pSS patients with parotid gland enlargement and anti-Ro/SSA and anti-La/SSB antibody positivity. Crevicular fluid analysis provides an easily accessible and representative site for the study of the oral microbiota in PSS. Certainly, further research is needed to fully understand the complex relationship between microbiota and autoimmunity.

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