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The microbiome in primary Sjögren's syndrome

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CHAPTER 3

Reduced salivary secretion contributes more to changes in the oral microbiome of primary Sjögren's syndrome patients than underlying disease

Adapted version of:

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INTRODUCTION

The oral microbiome of patients with primary Sjögren's syndrome (pSS) differs from that of healthy individuals (1–3). Whether these changes are the consequence of reduced salivary secretion or specific for pSS is yet unknown. Therefore, the aim of our study was to assess whether pSS patients have a specific oral microbiome compared to symptom controls (non-SS patients) and healthy controls (HCs).

METHODS

To capture the overall bacterial composition in the mouth, we collected oral washings from 121 consecutive patients referred for a diagnostic pSS workup and 14 volunteers without oral or ocular dryness (HCs). Patients fulfilling the 2016 ACR/EULAR classification criteria were classified as pSS (n=36) and patients not fulfilling the criteria as non-SS (n=85, see Table 1) (4).

Collection of oral samples

Patients and healthy controls were prohibited from eating, drinking other liquids than water, smoking and oral hygiene activities at least one hour before sampling. For the collection of an oral washing, the patient was instructed to rinse the oral cavity for 30 seconds with 10mL sterile water (Versylene Fresenius, Fresenius Kabi, France), without gargling. The water was saved in sterile 50mL tubes and the oral washing was spit back into this tube (#227261, Greiner Bio-One International, GmbH, Germany). Samples were put immediately on ice and stored at -80°C within 4 hours.

DNA isolation and sequencing

DNA isolation was performed with the UltraClean Microbial DNA isolation kit (MO BIO Laboratories, Carlsbad, California, USA). The bacterial composition was determined by Illumina MiSeq paired end sequencing of variable region V4 of the 16S rRNA gene. Each oral washing was vortexed for 5 seconds and 2mL of oral washing from two 1mL cryovials was transferred to a new single 2mL vial. This vial was spun at 10,000G for 30 seconds. The supernatant was discarded and the pellet was resuspended in 300 microliter MicroBead solution. Hereafter the manufacturer protocol was followed. Chemical lysis was obtained by using the provided solutions in the DNA isolation kit. Mechanical lysis was obtained by shaking the samples with MicroBead solution and microbeads in a tube on a flat vortex adapter (MO BIO, Carlsbad, California, USA) at full speed for 10 minutes at room temperature. DNA concentration was measured with a NanoDrop ND-1000 Spectrophotometer (Thermo

Fisher Scientific, Waltham, MA, USA). DNA was stored at -20°C before transport to the sequencing facility. Ten microliter of DNA solution was plated on 96 wells plates and transported to be sequenced at the Broad Institute, Cambridge, MA, USA.

Table 1: Patient characteristics*

Characteristic	Healthy controls 14	non-SS patients 85	pSS patients 36
Male gender, n (%)	1 (7.1)	18 (21.2)	5 (13.9)
Age, mean (sd)	41.7 (13.7)	54.9 (14.6)	59.1 (11.9)
Natural teeth present, n (%)	14 (100)	68 (88.3)	30 (88.2)
Smoking	0	16 (19.0)	0
Fulfilling 2016 ACR/EULAR criteria, n (%)	0 (0.0)	0 (0.0)	36 (100.0)
Xerostomia , n (%)	0 (0.0)	80 (94.1)	34 (94.4)
UWS mL/min, median (IQR)	0.51 (0.19)	0.13 (0.19)	0.07 (0.09)
SWS mL/min, median (IQR)	1.23 (0.6)	0.40 (0.47)	0.18 (0.34)
Hyposalivation (UWS≤0.1), n (%)	0 (0.0)	35 (41.2)	22 (61.1)
SSA positive, n (%)	NA	10 (11.8)	23 (63.9)
RA	0	4 (4.7)	3 (8.3)
SLE	0	0	0
Xerogenic medication	NA	47 (55.3)	17 (47.2)
PPI	NA	37 (43.5)	14 (38.9)
DMARD	NA	11 (12.9)	8 (22.2)
NSAID	NA	19 (22.4)	7 (19.4)
Methotrexate	NA	4 (4.7)	1 (2.8)
Antimalarial	NA	7 (8.2)	3 (8.3)
Corticosteroids	NA	2 (2.4)	4 (11.1)
Mean reads per sample (sd)	149568 (134936)	106729 (76116)	85105 (44158)

*pSS, primary Sjögren's syndrome; natural teeth present, any natural teeth present (i.e. not edentulous); xerostomia, symptoms of oral dryness; UWS/SWS, unstimulated/stimulated whole salivary secretion; hyposalivation, defined according to 2016 ACR/EULAR criteria; SSA, Sjögren's syndrome autoantibody A; RA, rheumatoid arthritis; SLE; systemic lupus erythematosus; xerogenic medication, using a drug with a reported moderate to high level of evidence of inducing salivary gland dysfunction xerostomia or subjective sialorrhea (12); PPI, proton pump inhibitor; DMARD, disease modifying antirheumatic drug; NSAID, non-steroid anti-inflammatory drug; sd, standard deviation; IQR, interquartile range. Age, UWS and SWS differed significantly between groups, while dental status (natural teeth present) was not significantly different.

Taxonomy determination and OTU filtering

Quantitative Insights In Microbial Ecology (QIIME) version 1.9.0 was used to cluster sequences with UCLUST version 1.2.22q at 97% similarity (5,6). Closed reference Operational Taxonomic

Unit (OTU) picking was performed against the Human Oral Microbiome Database (HOMD) (7). OTUs that were observed only once in a sample and OTUs with a relative abundance of <0.01% were filtered out. This strict filtering approach cleaned the OTU-table of rare and very low abundant OTUs. All samples were rarefied (i.e., equalized) to 8000 reads/sample.

Multivariate association with linear models (MaAsLin)

Comparative statistics were performed in R version 3.4.2 (8). MaAsLin is a statistical framework that performs boosted, additive general linear models between clinical data and bacterial abundance to find associations between clinical data (categorical or continuous) and bacterial taxa (<https://bitbucket.org/biobakery/maaslin/>) (9). MaAsLin was used to find associations between bacterial taxa and disease status (pSS, non-SS or HCs). Age, smoking and dental status were included in the statistical model to deconfound for these factors (10,11). MaAsLin calculates a false discovery rate (FDR) corrected p-value. A FDR p-value of <0.10 was used as significance cut-off.

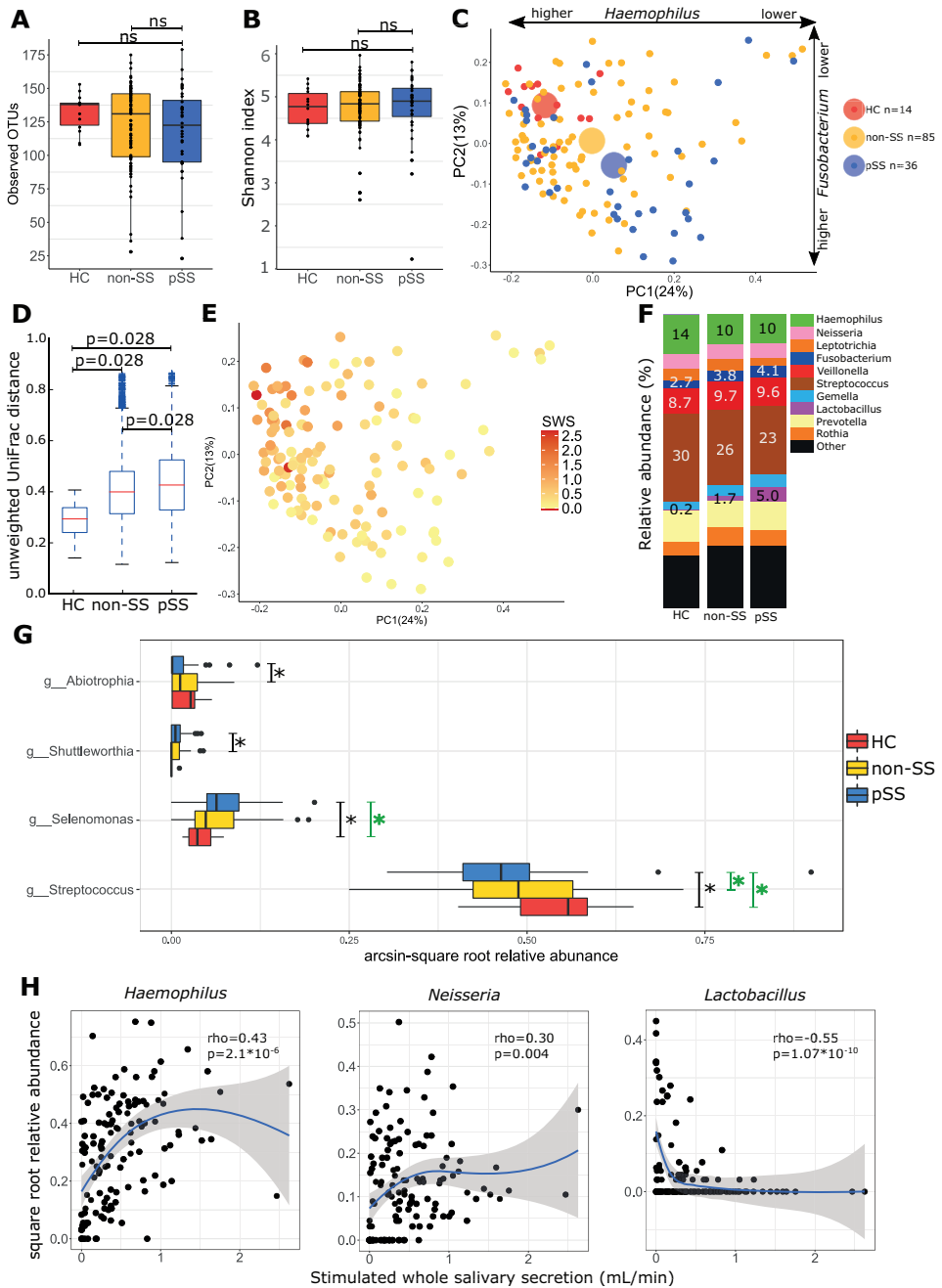
RESULTS AND DISCUSSION

Alpha-diversity, i.e., the number of bacterial taxa and proportion in which each taxon is represented per sample, was measured by the number of observed operational taxonomic units (OTUs) and Shannon index. Alpha-diversity was similar between pSS, non-SS and HCs, indicating that oral washings of pSS patients harbour a similar number of bacterial taxa and a similar distribution of relative abundances of taxa as non-SS patients and HCs (figures 1A,1B).

Beta-diversity, i.e., the dissimilarity between samples based on the relative abundance of bacterial taxa, showed a similarly large variation in bacterial composition in samples from pSS and non-SS patients compared to HCs (figures 1C,1D). This suggests that the oral microbiome in pSS and non-SS patients is more heterogeneous than that of HCs. This large heterogeneity in bacterial composition made it impossible to identify individual pSS patients based on overall oral bacterial composition. Disease status (pSS, non-SS or HCs) explained only 5% of the variation between samples ($p < 0.001$, Adonis), while stimulated whole salivary secretion (SWS) explained 9% (figure 1E) ($p < 0.001$). Despite the large variation between individual pSS patients, average overall bacterial composition in pSS patients differed from non-SS patients and HCs, located at highest principal coordinate (PC)1 and lowest PC2 (figures 1C,1F). *Haemophilus* and *Fusobacterium* were negatively correlated with PC1 and PC2, respectively, indicating that pSS patients have lower *Haemophilus* and higher *Fusobacterium* relative abundance on average.

Multivariate Association with Linear Models (MaAsLin) found lower *Streptococcus* and higher *Selenomonas* relative abundances significantly associated with pSS compared to HCs (false discovery rate corrected (FDR) $p < 0.10$, taking into account age, dental and smoking status)(figure 1G) (9–11). Non-SS patients showed more similar relative abundances of these genera. *Abiotrophia* and *Shuttleworthia* were associated with pSS compared to non-SS (figure 1G, Supplementary Table S1). Surprisingly, previous studies reported higher oral *Streptococcus* relative abundance in pSS patients compared to HCs (1–3). These studies used different samples (saliva, buccal and tongue swabs) and sequenced different variable regions, which may explain the discrepancy with our study (online supplementary Table S2) (1–3). *Streptococcus* relative abundance in our study was not correlated with SWS (Spearman, $p = 0.52$) and was significantly associated with pSS compared to HCs and non-SS independent of SWS (figure 1G, FDR $p < 0.10$). This suggests that lower *Streptococcus* relative abundance is more disease-specific than related to SWS. In contrast, *Haemophilus*, *Neisseria* and *Lactobacillus* relative abundance was significantly correlated with SWS, but not with disease status, indicating that lower SWS in pSS patients can explain changes of these genera (figures 1F,1H).

FIGURE 1: Characteristics of the oral microbiome in pSS patients, non-SS patients and HCs. (A,B) Alpha-diversity measured by the number of observed OTUs and Shannon index (ns = not significant, Wilcoxon test with Benjamini Hochberg correction for multiple comparisons, $\alpha \leq 0.05$). (C) Beta-diversity, showing the dissimilarity between individual samples (small dots) and between group means (large dots) in a principal coordinate analysis (PCoA) based on unweighted UniFrac distance. *Haemophilus* had the strongest (negative) correlation with PC1 (coefficient -0.45 , FDR $p = 1.6 \times 10^{-9}$) and *Fusobacterium* correlated strongly negatively with PC2 (coefficient -0.34 , FDR $p = 2.3 \times 10^{-13}$, Multivariate Associations with Linear Models (MaAsLin)). The smaller the distance between dots, the more similar the bacterial composition and vice-versa. At group level, pSS and non-SS differed significantly from HCs in PC1 ($p = 0.005$ and $p = 0.008$, respectively) and PC2 ($p = 0.003$ and $p = 0.008$, respectively), while pSS was significantly different from non-SS in PC2 ($p = 0.02$), but not in PC1 ($p = 0.17$, Wilcoxon test with Benjamini Hochberg correction). (D) Distance boxplot showing a significantly larger unweighted UniFrac distance between samples within pSS patients, followed by non-SS patients in comparison with HCs (two-sided Student's two sample t-test, Bonferroni corrected non-parametric p-values using 999 permutations). (E) PCoA as in (C), colored according to stimulated whole salivary secretion (SWS, mL/min). (F) Mean relative abundance of the ten most abundant genera per group. (G) Bacterial taxa significantly associated with pSS compared to HCs and non-SS patients, taking into account age, dental and smoking status (black bars) and additionally accounting for SWS (green bars)(MaAsLin, * FDR $p < 0.10$) (H) Three of the ten most abundant genera were significantly correlated with SWS(Spearman, Bonferroni corrected p-value). Blue line shows the locally weighted scatterplot smoothing line (non-parametric regression method) and in grey the 95% confidence interval.



CONCLUSION

We conclude that salivary secretion has a stronger influence on the microbiome in oral washings than disease status. However, lower *Streptococcus* relative abundance in pSS patients appears to be a disease-specific effect.

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SUPPLEMENTARY TABLES

TABLE S1: Bacterial genera significantly associated with pSS compared to HCs and non-SS patients*

Bacterial taxon	Groups compared	Coefficient	N	N not 0	P-value	Q-value
g__Selenomonas	pSS vs HCs	0.037138	50	48	0.001535	0.024717
g__Streptococcus	pSS vs HCs	-0.09457	50	50	0.008449	0.087668
g__Shuttleworthia	pSS vs non-SS	0.003449	121	41	0.007691	0.059678
g__Abiotrophia	pSS vs non-SS	-0.01263	121	59	0.009134	0.066846

*Analysis performed in MaAsLin, taking into account age, smoking and dental status. Only taxa with a significant association with pSS are shown (q-value < 0.10). g__ = genus. A positive coefficient indicates higher relative abundance in pSS patients and a negative coefficient indicates a lower relative abundance in pSS patients compared to either HCs or non-SS patients. N = total number of samples, N not 0 = number of samples in which the relative abundance of that taxon was higher than 0. Q-value is the false discovery rate (FDR) corrected p-value.

TABLE S2: Comparison results from the present study with previous studies on the oral microbiome in pSS patients versus healthy controls*

Study	Study population	Sample type and 16S region	Alpha-diversity	Beta-diversity	Bacterial taxa (phylum and genus level) higher in (p)SS compared to HCs	Bacterial taxa (phylum and genus level) lower in (p)SS compared to HCs	Comments
Van der Meulen et al.	36 pSS patients 14 HCs	Oral washings V4	Similar observed OTUs and Shannon diversity	No clear clustering of samples based on disease status was visible in the PCoA. Disease status explained 5% of the variation between samples.	Firmicutes (NS), Actinobacteria (NS), Selenomonas Fusobacterium (NS), Lactobacillus (NS), Gemella (NS), Veillonella (NS), Porphyromonas (NS)	Proteobacteria (NS), Streptococcus Haemophilus (NS), Leptotrichia (NS), Neisseria (NS), Granulicatella (NS)	Associations between pSS and bacterial taxa were corrected for age and dental status, with FDR correction. None of the pSS or HCs was smoker.
Van der Meulen et al.	36 pSS patients 85 non-SS patients	Oral washings V4	Similar observed OTUs and Shannon diversity	pSS and non-SS patients more similar to each other than to HCs.	Higher in pSS vs non-SS: <i>Shuttleworthia</i> , <i>Fusobacterium</i> (NS), <i>Lactobacillus</i> (NS)	Lower in pSS vs non-SS: <i>Abiotrophia</i> , <i>Streptococcus</i> , <i>Rothia</i> (NS), <i>Leptotrichia</i> (NS)	Associations between pSS and bacterial taxa were corrected for age, dental and smoking status, with FDR correction.
De Paiva et al.(2)	10 SS patients 11 HCs	Tongue swab V1-V3	Lower Shannon diversity in SS patients, similar observed OTUs	SS and HCs differed significantly and disease status was an important contributor to this difference	<i>Streptococcus</i>	<i>Leptotrichia</i> , <i>Fusobacterium</i> , <i>Bergeyella</i> , <i>Peptostreptococcus</i> , <i>Butyrivibrio</i>	No correction for multiple testing.
Li et al.(1)	10 pSS patients 10 HCs	Buccal mucosa V1-V3	Lower number of genera per sample in SS patients	<i>Haemophilus</i> , <i>Gemella</i> , <i>Neisseria</i> , <i>Lautropia</i> , <i>Rothia</i> more closely related to HCs. <i>Streptococcus</i> , <i>Lactobacillus</i> , <i>Tannerella</i> , <i>Treponema</i> , <i>Leptotrichia</i> more related to SS.	<i>Leucobacter</i> , <i>Delftia</i> , <i>Pseudochrobactrum</i> , <i>Ralstonia</i> , <i>Mitsuaria</i>	<i>Proteobacteria</i> , <i>Haemophilus</i> , <i>Neisseria</i> , <i>Comamonas</i> , <i>Granulicatella</i> , <i>Limnochlamydomonas</i>	No correction for multiple testing.
Siddiqui et al.(3)	9 pSS patients 9 HCs	Whole unstimulated saliva V1-V2	Lower observed OTUs and lower Shannon diversity in pSS (no statistical test was found)	PCoA shows 2 separate groups based on disease status using PC2 and PC3. (no statistical test was performed)	<i>Firmicutes</i> , <i>Streptococcus</i> , <i>Veillonella</i>	<i>Synergistetes</i> , <i>Spirochaetes</i> , <i>Bacteroidetes</i> (NS), <i>Proteobacteria</i> (NS), <i>Treponema</i> , <i>Moryella</i> , <i>Freitbacterium</i> , <i>Porphyromonas</i> , <i>Tannerella</i> , <i>Catonella</i> , <i>Haemophilus</i> (NS) and <i>Neisseria</i> (NS)	All patients had a salivation rate of >0.1mL/min. FDR correction applied.

*Results corresponding to our research are highlighted in green and results non-similar or opposite to our results are highlighted in red. Bacterial taxa higher or lower in pSS compared to HCs are all significant according to the statistics used in the study, except when indicated as NS (not significant). FDR = false discovery rate.

