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The microbiome in primary Sjögren's syndrome

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CHAPTER 4

Dysbiosis of the buccal mucosa microbiome in primary Sjögren's syndrome patients

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ABSTRACT

Objectives. Environmental factors in the aetiology of primary Sjögren's syndrome (pSS) are largely unknown. Host–microbiome interaction at mucosal surfaces is presumed to be involved in the etiopathogenesis of pSS. Here, we assessed whether the microbiome of the buccal mucosa is specific for pSS compared with symptom-controls.

Methods. The bacterial composition of buccal swab samples from 37 pSS patients, 86 non-SS sicca patients (with similar dryness symptoms as pSS patients, but not fulfilling the classification criteria) and 24 healthy controls (HCs) was determined with 16S rRNA sequencing. Multivariate Association with Linear Models (MaAsLin) was used to find associations between individual taxa and pSS, taking into account smoking and dental status. Associations were replicated in a general population cohort (n=103).

Results. The buccal mucosa microbiome of pSS and non-SS sicca patients differed both from HCs. Higher Firmicutes/Proteobacteria ratio was characteristic for both pSS and non-SS sicca patients. Disease status (pSS, non-SS sicca, HCs) and salivary secretion rate contributed almost equally to the variation in bacterial composition between individuals (3.8% and 4.3%, respectively). Two taxa were associated with pSS compared with non-SS sicca patients and 19 compared with HCs. When salivary secretion rate was taken into account, no taxon was associated with pSS compared with non-SS sicca. Twelve of the 19 pSS-associated taxa were correlated with salivary secretion.

Conclusion. Dysbiosis of the buccal mucosa microbiome in pSS patients resembles that of symptom-controls. The buccal mucosa microbiome in pSS patients is determined by a combination of reduced salivary secretion and disease-specific factors.

INTRODUCTION

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease characterized by chronic inflammation of the salivary and lacrimal glands resulting in complaints of dryness of mouth (xerostomia) and eyes (keratoconjunctivitis sicca). Extraglandular manifestations include arthritis, peripheral neuropathy, cutaneous vasculitis, respiratory dysfunction and tubulointerstitial nephritis (1–3). The prevalence of pSS ranges from 0.02 to 0.1% with women being affected 10 times more than men (4,5).

Genetic predisposition and environmental factors are the most important factors involved in the aetiology of pSS (6,7). Variants in HLA-genes and genes related to innate and adaptive immunity have been associated with Sjögren's syndrome, with major effect of HLA-genes (odds-ratio 3.5) and moderate effects of six non-HLA genetic loci (odds-ratios 0.7-1.6) (6). Besides genetic predisposition, familial factors also play an important role in the aetiology of pSS, although it is unknown what these familial factors are (8). Currently, there is still a large gap in knowledge on environmental factors involved in the aetiology of pSS.

Several studies have reported that the dysbiosis in the gut microbiome may contribute to the development of pSS (9–11). However, the role of the oral microbiome – the most proximal microbial habitat of the gastrointestinal tract – is largely unknown. Oral *Klebsiella* species can induce severe gut inflammation in mice, suggesting that the oral cavity may serve as a reservoir for potential intestinal opportunistic pathobionts (12). In humans, this reservoir may be intracellular bacteria in buccal epithelial cells (13,14). Shedding of buccal epithelial cells may transmit the intracellular bacteria to the gut. Furthermore, increasing evidence suggests a role for cross-reactivity of commensal oral and gut bacteria with Sjögren's syndrome antigen A (SSA)/Ro60 in the etiopathogenesis of pSS (15,16).

The oral microbiome in pSS patients differs from healthy controls, as was revealed in three relatively small studies including ≤ 10 pSS patients (10,17,18). We recently reported that the bacterial composition in oral washings from pSS patients and symptom-controls showed similar changes compared with HCs and that lower *Streptococcus* relative abundance was associated with pSS (19). However, whether the buccal mucosa microbiome is specific for pSS is currently unknown.

Therefore, we compared the buccal mucosa microbiome of pSS patients with that of symptom-controls (i.e., patients suspected for pSS not fulfilling the pSS classification criteria: non-SS sicca patients) and healthy controls (HCs). Furthermore, we measured salivary

secretion rate to assess its influence on the buccal mucosal microbiome. Finally, association between bacterial taxa and pSS were tested for significance in a replication cohort of individuals from the general population.

METHODS

Study participants

In a case-control design, we collected 158 buccal swab samples, of which 134 from consecutive patients referred for a diagnostic pSS work-up and 24 from HCs. Eligible participants were ≥ 18 years old and were recruited between June 2015 and March 2017. Patients who did not complete the diagnostic pSS work-up were excluded. Patients fulfilling the 2016 ACR/EULAR classification criteria were classified as pSS and patients not fulfilling the criteria were classified as non-SS sicca (20). HCs did not have oral dryness complaints or rheumatic disease and matched with pSS patients on sex, but not on age. Age and sex have not been shown to influence the oral microbiome composition (21). The study was approved by the medical ethical committee of the University Medical Center Groningen, Groningen, the Netherlands (METc 2015/211). All participants completed written informed consent according to the declaration of Helsinki.

Data on age, sex, smoking, medication use and EULAR Sjögren's syndrome patient reported index (ESSPRI) were collected from medical history records. Unstimulated and stimulated whole saliva (UWS and SWS, respectively) was collected from all pSS patients, non-SS sicca patients and HCs. Salivary secretion rate was measured in ml/min.

Smoking was defined as smoking at least one cigarette per day. Xerogenic medication was defined as using a drug within the chemical subgroup (level 4 of the Anatomical Therapeutic Chemical classification) with a reported moderate to high level of evidence of inducing salivary gland dysfunction, xerostomia and subjective sialorrhea (22).

Replication cohort

Individuals from the general population ($n=103$), participating in a population based cohort study (LifeLines) and included in the LifeLines-DEEP project, were used as replication cohort (23). Metadata on age, sex and smoking status were available. No selection was performed on age or sex in order to represent the general population most accurately. Population controls were used to test whether associations of bacterial taxa with pSS compared with HCs could be replicated in the general population.

Sample collection, DNA isolation and sequencing

Patients referred for the diagnostic pSS work-up and HCs were prohibited from eating, drinking other liquids than water, smoking and oral hygiene activities at least one hour before sampling. From each patient or control, a buccal swab was collected (Eswab, COPAN, Italy). Buccal swabs from population controls were taken with sterile cotton swabs (Peha Hartmann, Paul Hartmann BV, the Netherlands) during a routine visit as part of the LifeLines population cohort study (23). DNA isolation on all samples was performed with the UltraClean Microbial DNA isolation kit (MOBIO, Carlsbad, California, USA) using mechanical and chemical lysis. Bacterial composition was determined by Illumina MiSeq paired end sequencing of variable region V4 of the 16S rRNA gene. See Supplementary Methods and Supplementary Figures for further details.

Taxonomy determination and filtering

Quantitative Insights In Microbial Ecology (QIIME) v1.9.1 was used to demultiplex and cluster sequences with UCLUST version 1.2.22q at 97% similarity (24,25). Closed reference Operational Taxonomic Unit (OTU) picking was performed against the Human Oral Microbiome Database (HOMD v14.51) (26). OTUs observed once in a sample and OTUs with a relative abundance of <0.01% were filtered out. Each OTU count was normalized to the total sum of counts in a sample. Of 158 buccal swab samples, 11 contained less than 1000 clustered reads/sample and were excluded from further analysis because of insufficient read depth. The remaining 147 samples contained a median 7086 reads/sample (interquartile range 3297-339653 reads/sample). All samples were rarefied (i.e., equated) to 1000 reads/sample in order to obtain equal sequencing depth.

Statistical analysis

QIIME was used to determine alpha-diversity (i.e., the bacterial diversity within one sample) and beta-diversity (i.e., the dissimilarity between samples based on overall bacterial composition). Alpha-diversity consists of richness (i.e., the abundance of observed taxa, measured as the number of observed OTUs) and evenness (i.e., how the relative abundance is distributed among taxa). Richness and evenness combined is measured by the Shannon index. The non-parametric Wilcoxon rank sum test was used to test group differences. Beta-diversity was measured with Bray-Curtis distance and visualized in a principal coordinate analysis (PCoA). A two sided Student's two-sample t-test with Bonferroni correction and 999 permutations was used to test whether the mean distance between individual samples within one study group differed from the other study groups. *Adonis* function from the *R-vegan* package in QIIME was used to describe the explained variance (R^2 -value) and significance (p-value) of clinical data on determining the variation in bacterial composition between samples using 999 permutations (27).

R v3.4.2 was used for comparative statistics (28). Benjamini and Hochberg false discovery rate (FDR) corrected p-values (q-value) were calculated for all comparative tests unless otherwise specified. A disease prediction model was built using Lasso method, with 5-fold cross-validation to estimate the prediction accuracy of the oral microbiome.

Multivariate Association with Linear Models (MaAsLin) was used to find associations between bacterial taxa and pSS (29). A q-value <0.10 was used as significance cut-off. A q-value between 0.10 and 0.20 was used to detect suggestive signals.

RESULTS

Bacterial community composition of the buccal mucosa in pSS patients resembles that of non-SS sicca patients

Samples from 37 classified pSS patients, 86 non-SS sicca patients and 24 HCs were used for analyses. On average, pSS patients were somewhat older, but the known important factors dental status and proportion of females did not differ significantly between pSS patients and HCs (Table 1). pSS and non-SS patients matched on age and sex. Salivary secretion rates were significantly lower in pSS patients compared with non-SS sicca patients and HCs ($p < 0.001$). Salivary secretion rates in non-SS sicca patients were also lower than in HCs ($p < 0.001$). Baseline characteristics are summarized in Table 1.

Profiles of overall bacterial relative abundance at phylum-level showed that the bacterial compositions of pSS and non-SS sicca patients were comparable and both differed from that of HCs (Figure 1A). A higher Firmicutes/Proteobacteria (F/P) ratio was characteristic for both pSS and non-SS sicca patients compared with HCs ($p = 0.01$, Wilcoxon, Figure 1B), suggesting that a high F/P ratio may be characteristic for the oral microbiome of oral dryness patients. At genus-level, similarities between pSS and non-SS sicca were less evident. Lower *Streptococcus* and higher *Gemella* relative abundances in pSS patients were the largest difference compared with non-SS sicca patients (Figure 1A).

Bacterial richness (observed OTUs) and diversity (Shannon index) did not significantly differ between pSS patients, non-SS sicca patients and HCs (Figure 1C). However, pSS and non-SS sicca patients showed a trend towards lower richness and diversity compared with HCs.

TABLE 1: Characteristics of pSS and non-SS sicca patients, HCs and population controls*

Characteristic	pSS patients n = 37	Non-SS sicca patients n = 86	HCs n = 24	Population controls n = 103	p-value**
Fulfilling 2016 ACR/EULAR criteria, %	100	0	NA	NA	NA
Female sex, n (%)	31 (84)	69 (81)	16 (83)	50 (49) ¹	0.9
Age in years, mean (sd)	59 (13)	56 (15)	42 (15)	55 (14)	<0.001
Smoking, n (%)	1 (3)	19 (22)	0	16 (16)	0.002
Own teeth, n (%)	29 (88)	68 (87)	24 (100)	58 (69)	0.2
Oral dryness, (%)	36 (97)	81 (94)	0	10 (15)	0.8
ESSPRI, mean	6	NA	NA	NA	NA
dryness, mean	7	NA	NA	NA	NA
fatigue, mean	6	NA	NA	NA	NA
pain, mean	6	NA	NA	NA	NA
SSA positive, n (%)	26 (70)	12 (16)	NA	NA	<0.001
SSB positive, n (%)	16 (43)	5 (7)	NA	NA	<0.001
RA, n (%)	3 (8)	5 (6)	0	NA	0.7
SLE, n (%)	0	0	0	NA	NA
SpA, n (%)	0	1 (1)	0	NA	NA
UWS (mL/min), median (IQR)	0.06 (0.02, 0.11)	0.13 (0.05, 0.25)	0.51 (0.41, 0.61)	NA	0.04
SWS (mL/min), median (IQR)	0.16 (0.01, 0.37)	0.37 (0.21, 0.69)	1.23 (1.03, 1.63)	NA	0.09
Hyposalivation (UWS<0.1mL/min), n (%)	23 (62)	34 (40)	0	NA	0.03
Xerogenic medication, n (%)	20 (54)	49 (57)	NA	NA	0.9
PPI, n (%)	16 (43)	39 (45)	NA	NA	1
DMARD, n (%)	8 (22)	11 (13)	NA	NA	0.3
NSAID, n (%)	6 (16)	20 (23)	NA	NA	0.5
Methotrexate, n (%)	1 (3)	4 (5)	NA	NA	1
Antimalarial agent, n (%)	3 (8)	7 (7)	NA	NA	1
Corticosteroids, n (%)	5 (14)	2 (2)	NA	NA	0.03

*pSS = primary Sjögren's syndrome; non-SS sicca = patients with a clinical suspicion of pSS not fulfilling the criteria for pSS, HCs = healthy controls. ACR/EULAR = American College of Rheumatology/European League Against Rheumatism; own teeth = any number of teeth present; oral dryness = according to 2016 ACR/EULAR subjective criteria; ESSPRI = EULAR Sjögren's syndrome patient reported index, based on visual analogue scale 1-10; SSA/SSB = Sjögren's syndrome autoantibody A/B; RA = rheumatoid arthritis; SLE = systemic lupus erythematosus, SpA = axial spondylitis; UWS/SWS = unstimulated/stimulated whole salivary flow rate; xerogenic medication = using a drug with a reported moderate to high level of evidence of inducing salivary gland dysfunction, xerostomia and subjective sialorrhea(22); PPI = proton pump inhibitor; DMARD = disease modifying antirheumatic drug; NSAID = non-steroid anti-inflammatory drug; NA = not applicable; sd = standard deviation.

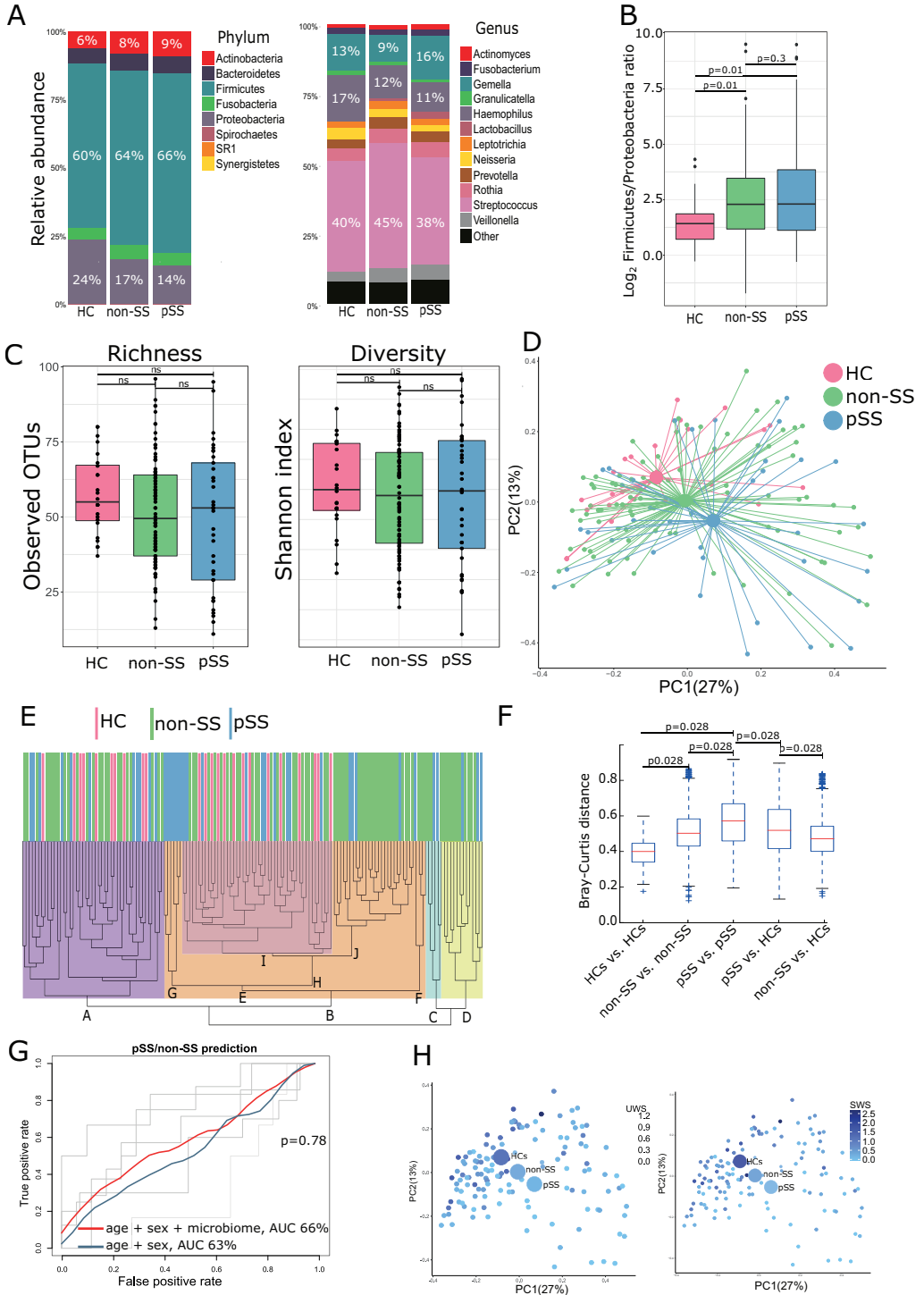
**p-values were calculated comparing pSS, non-SS sicca and HCs or comparing pSS and non-SS sicca when data for HCs was '0' or 'NA', using appropriate tests for data distribution (χ^2 test, one-way ANOVA, Fisher's exact test or Kruskal-Wallis test).

¹=Sex was significantly different between groups including population controls (p<0.001).

PCoA showed no evident clustering of individual samples according to disease status (pSS, non-SS sicca or HCs) (Figure 1D). However, centroids of pSS and non-SS sicca patients differed significantly from HCs in principal coordinate 1 (PC1) ($q=0.002$ and $q=0.0005$, respectively, Wilcoxon). The centroids of pSS and non-SS sicca patients were not significantly different ($q=0.25$ in PC1, $q=0.49$ in PC2). In the clustering dendrogram, samples from HCs clustered in clusters A and I, together with samples from pSS and non-SS sicca patients (Figure 1E). This suggests that pSS and non-SS sicca patients in clusters A and I have an oral bacterial community composition similar to HCs, while pSS and non-SS sicca patients in the other clusters have a more disease-related oral bacterial composition. pSS patients showed the largest variation in bacterial community composition between individuals, followed by non-SS sicca patients compared with HCs (Figure 1F). This indicates a very heterogeneous buccal mucosa microbiome in pSS patients and, to a lesser extent, in non-SS sicca patients. The large heterogeneity in pSS and non-SS sicca patients probably explains why it was not possible to predict pSS in a population with symptoms of oral dryness (pSS and non-SS sicca together) using differentially abundant genera in the buccal mucosa microbiome (Figure 1G).

Disease status explained a relatively low 3.8% of the variation in distances between individual samples in the Bray-Curtis distance matrix ($p<0.001$, *adonis*). Previously we showed that reduced salivary secretion contributes more to the microbiome of oral washings from pSS patients than underlying disease (19). In this study, UWS and SWS secretion rates explained 4.1% and 4.3% of the variation between individual buccal swab samples, respectively (Figure 1H, $p<0.001$). This suggests that disease status and salivary secretion have a similarly large effect on the buccal mucosa bacterial composition of an individual. Age explained 3.8% of the variation between all individual samples across all study groups ($p=0.002$). This indicates that the effect of age on comparisons between HCs and pSS/non-SS patients was very limited, because mean age differed relatively little between HCs and pSS/non-SS patients.

Figure 1: Characteristics of the buccal mucosa microbiome from pSS patients, non-SS sicca patients and HCs. **(A)** Profiles of the relative abundance at phylum and genus-level per group. **(B)** \log_2 transformed Firmicutes/Proteobacteria ratio. **(C)** Richness and Shannon diversity, ns = not significant. **(D)** Bray-Curtis based PCoA. Small dots are individual samples, large dots the centroids per group. **(E)** Bray-Curtis based clustering dendrogram. Each coloured line represents an individual, letters indicate clusters. **(F)** Bray-Curtis distance between individual samples from pSS patients (pSS vs. pSS), non-SS sicca patients (non-SS vs. non-SS) and HCs (HCs vs. HCs) **(G)** Prediction model for pSS, AUC = area under the curve. **(H)** Gradient in UWS and SWS secretion rate in PCoA.



In summary, the overall bacterial community composition on the buccal mucosa of pSS patients resembles that of non-SS sicca patients, while both pSS and non-SS sicca patients differ from HCs. Due to the large variation in buccal mucosa microbiome composition in pSS and non-SS sicca patients, bacterial genera in the buccal mucosa microbiome cannot be used to distinguish pSS from non-SS sicca. Disease status and salivary secretion contribute almost equally to the variation in bacterial composition on the buccal mucosa between all individuals.

Associations of individual bacterial taxa with pSS

Associations of individual bacterial taxa with pSS were analysed using MaAsLin, taking into account the possible confounding factors smoking and dental status (29–31). All taxonomic levels were analysed (Supplementary Table 1), but only phylum and genus-level are reported here for clarity (Table 2). Proteobacteria relative abundance was significantly lower in pSS patients compared with HCs ($q=0.082$). Non-SS sicca patients also showed lower relative abundances of Proteobacteria compared with HCs (17% vs. 24%), although this was not statistically significant.

At genus-level, 11 genera were significantly associated with pSS compared with HCs ($q<0.10$) and 7 genera showed a suggestive signal for association ($q=0.10-0.20$) (Figure 2, Table 2). When SWS secretion rate was taken into account on top of dental and smoking status, 8 taxa (among which: Haemophilus, Neisseria, Lautropia and Lactobacillus) lost its association with pSS (Supplementary Table 2), suggesting that SWS secretion rate has a large influence on the relative abundance of these taxa. Eleven other taxa (among which: Proteobacteria, Granulicatella and Bergeyella) remained significantly associated with pSS when SWS secretion rate was taken into account. This suggests a disease-specific connection between pSS and these taxa.

Compared with non-SS sicca patients, pSS was suggestively associated with lower relative abundances of Granulicatella and Bergeyella ($q=0.10$ and $q=0.15$, respectively) (Figure 2, Table 2). However, when SWS secretion rate was taken into account, no bacterial taxon remained associated with pSS compared with non-SS sicca. This suggests that no individual bacterial taxon is truly specific for pSS. Presence of anti-SSA or anti-SSB auto-antibodies was not associated with any bacterial taxon.

Replication of associations between bacterial genera and pSS in a general population cohort

The 18 genera associated with pSS compared with HCs were subsequently analysed with MaAsLin in comparison with population controls ($n=103$), taking into account sex, dental and smoking status. Granulicatella, Abiotrophia and Ruminococcaceae (all belonging to

phylum Firmicutes) and Lautropia (belonging to phylum Proteobacteria), were significantly lower in pSS compared with population controls ($q < 0.10$, Figure 2, Table 2). However, the same taxa were significantly associated with non-SS sicca patients compared with population controls ($q < 0.10$, Supplementary Table 1). This suggests that lower relative abundances of Abiotrophia, Ruminococcaceae, Lautropia and Granulicatella are more likely to be associated with dysbiosis in the oral microbiome of oral dryness patients than being specific for pSS.

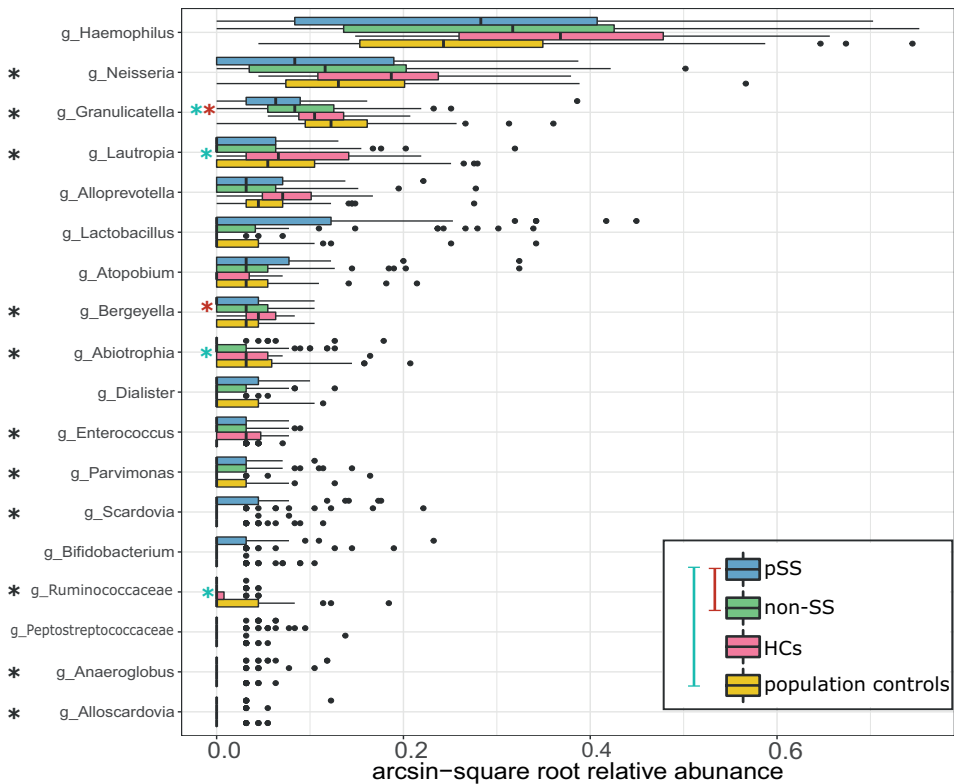


FIGURE 2: Genera (g_) associated with pSS compared with HCs taking into account smoking and dental status. Genera are ordered by overall mean relative abundance. X-axis represents the arcsin-square root transformed relative abundance. Genera significantly associated with pSS compared with HCs ($q < 0.10$) are indicated by a black asterisk, genera not indicated showed a suggestive signal for association ($q = 0.10-0.20$) (see Table 2). Red asterisks indicate genera associated with pSS compared with non-SS sicca. Blue asterisks indicate genera associated with pSS compared with population controls.

TABLE 2: Bacterial taxa associated with pSS compared with non-SS sicca, HCs and population controls*

Bacterial taxon	Groups compared	Coefficient	↓/↑ in pSS	N	N not 0	p-value	q-value
p_Actinobacteria g_Alloscardovia	pSS vs HCs	0.008	↑	61	7	0.006	0.082
p_Actinobacteria g_Bifidobacterium	pSS vs HCs	0.027	↑	61	14	0.012	0.103
p_Actinobacteria g_Scardovia	pSS vs HCs	0.033	↑	61	15	0.008	0.089
p_Actinobacteria g_Atopobium	pSS vs HCs	0.023	↑	61	31	0.021	0.119
p_Bacteroidetes g_Alloprevotella	pSS vs HCs	-0.029	↓	61	40	0.024	0.124
p_Bacteroidetes g_Bergeyella	pSS vs HCs	-0.023	↓	61	37	0.003	0.080
p_Bacteroidetes g_Bergeyella	pSS vs non-SS sicca	-0.015	↓	123	75	0.016	0.151
p_Firmicutes g_Abiotrophia	pSS vs HCs	-0.022	↓	61	21	0.001	0.057
p_Firmicutes g_Abiotrophia	pSS vs Pop. Cont.	-0.024	↓	140	67	0.001	0.023
p_Firmicutes g_Granulicatella	pSS vs HCs	-0.052	↓	61	53	0.000	0.013
p_Firmicutes g_Granulicatella	pSS vs non-SS sicca	-0.031	↓	123	108	0.004	0.100
p_Firmicutes g_Granulicatella	pSS vs Pop. Cont.	-0.066	↓	140	129	1.8*10 ⁻⁷	1.3*10 ⁻⁵
p_Firmicutes Enterococcus	pSS vs HCs	-0.021	↓	61	29	0.003	0.080
p_Firmicutes g_Lactobacillus	pSS vs HCs	0.010	↑	61	20	0.032	0.159
p_Firmicutes g_Parvimonas	pSS vs HCs	0.016	↑	61	20	0.010	0.093
p_Firmicutes g_Peptostreptococcaceae	pSS vs HCs	0.010	↑	61	11	0.022	0.119
p_Firmicutes g_Ruminococcaceae	pSS vs HCs	-0.009	↓	61	7	0.010	0.094
p_Firmicutes g_Ruminococcaceae	pSS vs Pop. Cont.	-0.014	↓	140	40	0.002	0.043
p_Firmicutes g_Anaeroglobus	pSS vs HCs	0.020	↑	61	9	0.005	0.082
p_Firmicutes g_Dialister	pSS vs HCs	0.012	↑	61	16	0.027	0.139
p_Proteobacteria	pSS vs HCs	-0.157	↓	61	58	0.006	0.082
p_Proteobacteria g_Lautropia	pSS vs HCs	-0.046	↓	61	35	0.004	0.080
p_Proteobacteria g_Lautropia	pSS vs Pop. Cont.	-0.043	↓	140	87	0.002	0.043
p_Proteobacteria g_Neisseria	pSS vs HCs	-0.088	↓	61	46	0.006	0.082
p_Proteobacteria g_Haemophilus	pSS vs HCs	-0.128	↓	61	58	0.019	0.119

*Analysis performed in MaAsLin, taking into account smoking and dental status. Bacterial taxonomy is shown at phylum (p_) and genus (g_) level; Pop.Cont. = population controls; Coefficient = difference of the arcsin-square root transformed relative abundance between groups, negative indicates lower in pSS, positive higher in pSS, clarified by arrows in the adjacent column; N = total number of samples used for the comparison; N not 0 = samples in which the relative abundance of the taxon was not 0; q-value = false discovery rate (FDR) corrected p-value.

Salivary secretion partially explains dysbiosis in the oral microbiome of pSS and non-SS sicca patients

To further explore the role of salivary secretion rate on the buccal mucosa microbiome, we tested correlations of UWS and SWS secretion rates with alpha-diversity, first two principal coordinates, four most abundant phyla, 12 most abundant genera and 19 pSS-associated taxa (Figure 3A). Alpha-diversity was not significantly correlated with UWS or SWS secretion rates, suggesting that bacterial richness and diversity of the buccal microbiome are not directly affected by the amount of salivary secretion. SWS secretion rate correlated mostly stronger than UWS secretion rate with the same bacterial taxa. SWS secretion rate was moderately strong correlated with phylum Proteobacteria and the corresponding genera *Haemophilus*, *Lautropia* and *Neisseria*, as well as with *Alloscardovia*, *Granulicatella* and *Lactobacillus* (Spearman's $\rho=0.40-0.51$, $q<0.01$) (Figure 3A-B). Weaker correlations were found between SWS secretion rate and *Bifidobacterium*, *Atopobium*, *Alloprevotella*, *Bergeyella* and *Enterococcus* (Spearman's $\rho=0.13-0.37$, $q<0.01$). Bacterial taxa with a positive correlation with SWS corresponded with a lower observed relative abundance in pSS patients and vice versa (Figures 2 and 3). In total, 12 of the 19 bacterial taxa associated with pSS compared with HCs were significantly correlated with SWS ($q<0.01$). This suggests that a substantial part of differentially abundant bacterial taxa in the oral microbiome of pSS patients can be explained by lower salivary secretion rate compared with HCs. However, correlations were weakly to moderately strong, which indicates that disease-specific factors also play a role in determining the relative abundance of these taxa. Indeed, phylum Proteobacteria and genera *Granulicatella*, *Abiotrophia*, *Enterococcus*, *Bergeyella* and *Bifidobacterium*, which were all correlated with SWS, also remained associated with pSS when SWS secretion rate was accounted for (Supplementary Table 2).

Interestingly, high abundant phyla, such as Firmicutes and Actinobacteria and high abundant genera, such as *Streptococcus*, *Gemella* and *Veillonella*, were not correlated with salivary secretion rates. Although none of these taxa were significantly associated with pSS, they differed in relative abundance between pSS, non-SS and HCs (Figure 1A). This suggests that disease-specific factors, or other factors not accounted for in this study, determine the relative abundance of these taxa. Salivary mucin concentration, antimicrobial peptides or the coexistence of other bacteria are several possible explanatory factors (32–34). Indeed, *Veillonella* relative abundance was negatively correlated with *Streptococcus* and *Gemella* relative abundances (Spearman's $\rho=-0.60$ $q=4.3*10^{-9}$ and $\rho=-0.67$ $q=8.6*10^{-14}$, respectively), suggesting a competitive relation between these genera (Supplementary Figure 6).

ESSPRI and dryness severity were not correlated with UWS, SWS, microbial diversity or any bacterial taxon, indicating that severity of dryness symptoms, as observed by the patient, is independent of the actual level of salivary secretion and bacterial community composition on the buccal mucosa.

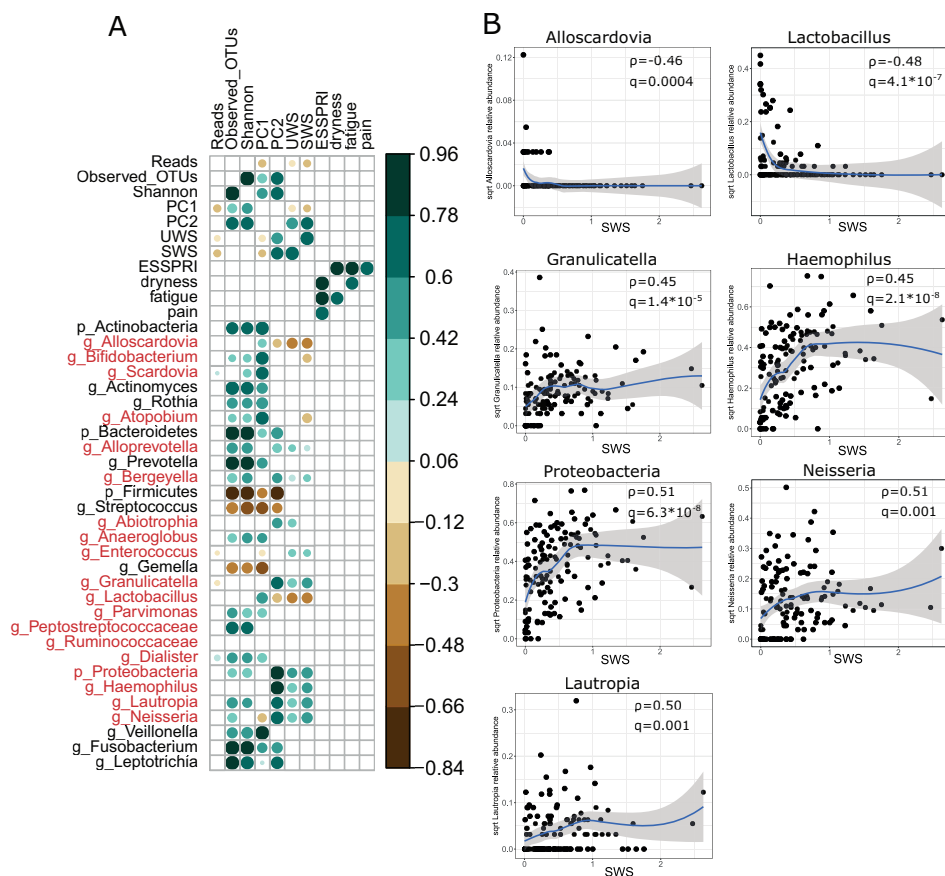


FIGURE 3: Correlations between salivary secretion and other parameters with bacterial taxa. **(A)** Each coloured dot indicates a significant correlation (Spearman's, $q < 0.01$). p_{-} = phylum, g_{-} = genus. Colour and size of the dots correspond with the direction and strength of the correlation. Taxa coloured in red are associated with pSS compared with HCs. **(B)** Correlations of individual bacterial taxa with SWS (Spearman's $\rho > 0.40$). Y-axis represents the square root relative abundance. Blue curves indicate the local regression fits (loess, non-parametric regression) with shaded in grey the 95% confidence interval.

DISCUSSION

The results of our study show that the buccal mucosa microbiome of pSS patients and non-SS sicca patients differ from that of HCs. Small differences were observed between pSS and non-SS sicca patients at population level, but the buccal mucosa microbiome was unable to distinguish pSS patients from non-SS sicca patients. No individual bacterial taxon was significantly higher in pSS patients compared with symptom-controls, which makes it unlikely that an individual bacterial taxon in the buccal mucosa microbiome is an etiological factor for pSS.

Richness and Shannon diversity were not significantly different between pSS, non-SS sicca and HCs. In contrast, other studies reported a significantly lower richness and Shannon diversity in saliva and tongue swabs from pSS patients than from HCs (10,17). However, only 10 pSS patients or less were included in these studies, which may not have been a representative sample of the total pSS population. Furthermore, we included recently diagnosed pSS patients, which may have resulted in a more similar alpha-diversity as in HCs, because disease duration and/or hyposalivation have not lasted long enough to affect the alpha-diversity of the buccal mucosa microbiome (35).

The larger heterogeneity in buccal mucosa bacterial composition in pSS patients may be caused by heterogeneity in the pSS population, especially in terms of differences in salivary secretion rate. We show that salivary secretion rate explains an almost equal percentage of the variation in bacterial composition as disease status. We and others have recently reported a similar effect of salivary secretion rate on the microbiome in oral washing samples and combined oral cavity samples (19,36). In this study, we could explain 3.8% of the individual variation in bacterial composition by disease status and 4.1-4.3% by salivary secretion rate. Host genetics, dentition state and environmental factors, such as household, medication, diet and oral hygiene may also contribute to the variation in bacterial composition of the buccal mucosa (30,37-39).

Also at the level of individual taxa, we show that both salivary secretion rate and pSS-specific factors affect relative abundance. Especially phylum Proteobacteria and its genera *Haemophilus*, *Lautropia* and *Neisseria* appear to be sensitive to the amount of saliva in the oral cavity. Interestingly, a species within phylum Proteobacteria (i.e., *Pseudomonas aeruginosa*) is sensitive to dryness and may therefore not survive on dry (mucosal) surfaces (40). Thus, lower relative abundance of Proteobacteria in pSS and non-SS sicca patients may be explained by a more dry mucosal surface compared with HCs. However, since phylum Proteobacteria remained associated with pSS compared with HCs when SWS secretion rate was accounted for, a pSS-specific effect may also contribute to its relative abundance.

Lactobacillus relative abundance was mostly influenced by salivary secretion rate. Lactobacillus is a well-known pathogen involved in dental caries (41), of which pSS patients indeed have a higher burden than healthy controls (42). No higher relative abundance of periodontitis associated taxa was observed in pSS patients. This corresponds with a similar risk of periodontitis in pSS patients as in HCs (43). Lower relative abundances of phylum Proteobacteria and genera Haemophilus, Neisseria, Granulicatella and Lautropia and higher relative abundances of Firmicutes and genera Lactobacillus, Atopobium, Parvimonas and Scardovia have previously been reported in pSS patients compared with HCs (17–19,36). However, we are the first to show that both pSS-specific factors and reduced salivary secretion contribute to the relative abundance of a large proportion of these taxa on the buccal mucosa.

The correlations between taxa and salivary secretion rate we report in our study are important for interpreting earlier studies that report associations between oral bacteria and diseases. For example, higher oral Lactobacillus and lower Haemophilus and Neisseria relative abundances have been associated with RA (44). However, these associations may be caused by lower salivary secretion rate, which has been reported in RA patients (45).

We conclude, that dysbiosis of the buccal mucosa microbiome is present in both pSS patients and symptom-controls when compared with HCs. The microbiome of the buccal mucosa is not specific enough for pSS and therefore not useful for characterizing pSS patients in clinical practice. The buccal mucosa microbiome in pSS patients is influenced by reduced salivary secretion rate and currently unknown disease-specific factors. Furthermore, our results suggest that it is unlikely that one specific bacterial taxon in the buccal mucosa microbiome is involved in the aetiology of pSS. Nevertheless, persistent dysbiosis in the buccal mucosa microbiome, combined with chronically reduced salivary secretion, may induce dysbiosis in the gut microbiome (9,12,14). Future studies assessing both the oral and gut microbiome might elucidate more of the relationship between the oral and gut microbiome and pSS.

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SUPPLEMENTARY METHODS

Buccal swab collection and preservation

After collecting buccal swabs from pSS, non-SS sicca and HCs, the swab was placed back in the provided tube with 1 mL liquid Amies transport medium. Swabs were directly placed on ice and were stored at -80°C within 4 hours after sampling. Buccal swabs from the population controls were saved in 300 µl MicroBead solution (from the UltraClean Microbial DNA Isolation Kit from MO BIO Laboratories Inc, Carlsbad, CA, USA) and stored at -80°C.

DNA isolation

DNA isolation of all buccal swabs (pSS, non-SS sicca, HCs and population controls) was performed with the UltraClean Microbial DNA Isolation Kit (cat.12224) from MO BIO Laboratories, Inc. (MO BIO, Carlsbad, California, USA). After thawing at room temperature with gentle shaking, the tubes with the swabs were vortexed for 30 seconds to maximize the release of bacterial contents into the preservation fluid. The swab was removed from the tube and the preservation fluid was transferred to a 2mL vial. This vial with either 1mL Amies solution or 300 µl Microbead solution was spinned at 10.000G for 30 seconds. The supernatant was discarded and the pellet was resuspended in 300 microLiter MicroBead solution. Hereafter the protocol was followed as provided by the manufacturer. 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. 45 µl of DNA solution was plated on 96 wells plates and transported to the sequence facility (Broad Institute, Cambridge, MA, USA).

Sequencing/ 16S rRNA profiling and sequencing. The 16S gene dataset consists of Illumina MiSeq sequences targeting the V4 variable region. Genomic DNA was subjected to 16S amplifications using primers designed incorporating the illumina adapters and a sample barcode sequence, allowing directional sequencing covering variable region V4 (Primers: 515F [GTGCCAGCMGCCGCGGTAA] and 806R [GGACTACHVGGGTWTCTAAT]). Sample concentrations were normalized to 1.5ng/µl. PCR mixtures contained 10 µl of sample template, 10 µl of 5PRIME HotMasterMix (Quantabio, Beverly, MA, USA), and 5 µl of primer mix (IDT, Coralville, IA, USA) at 2 µM concentration of each primer. The cycling conditions consisted of an initial denaturation of 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 45 sec, annealing at 50 °C for 60 sec, extension at 72°C for 5 min, and a final extension at 72°C for 10 min. Amplicons were quantified according to the Caliper LabChip GX:

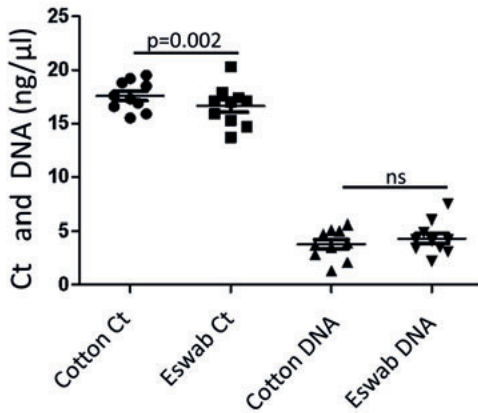
DNA 5K Assay (PerkinElmer, Hopkinton, MA, USA), and pooled in equimolar concentrations according to the 400bp amplicon. The pooled samples were then size selected by Pippin Prep 2% agarose protocol (Sage Science, Beverly, MA, USA), removing non-specific amplification products from host DNA (non 375-425 bp). Sequencing was performed on the Illumina MiSeq v2 platform, according to the manufacturer's specifications with addition of 5% PhiX, and generating paired-end reads of 175b in length in each direction. Paired end read alignment and quality control was performed in QIIME.

Effect of swab type and preservation fluid on bacterial composition

The swab type and preservation fluid used in our study cohort (pSS patients, non-SS sicca patients and HCs) differed from that used in the general population replication cohort. In our study cohort, flocked swabs (Eswab, COPAN, Italy) were used and preserved in the provided Liquid Amies Elution. In the replication cohort, cotton swabs were used (Peha Hartmann, Paul Hartmann BV, the Netherlands) and swabs were preserved in 300 μ L Microbead solution from the UltraClean Microbial DNA isolation kit (MO BIO Laboratories, Carlsbad, California, USA). Although all subsequent steps regarding DNA isolation and 16S sequencing were identical in both cohorts, we were interested whether swab type and preservation fluid had an effect on the bacterial composition in these samples. Therefore, we tested these two techniques in 10 healthy individual volunteers. After signing informed consent, one side of the buccal mucosa was swabbed with the flocked Eswab and the other side with the cotton swab. Swabs were then placed in their respective preservation fluids as described above and stored at -80°C . DNA isolation was performed as described above. DNA concentration in the DNA isolates was measured and a quantitative polymeric chain reaction was performed with a Bact338 primer (Biolegio BV, Nijmegen, the Netherlands). No significant difference between the two swab types was observed in DNA concentration, but the cycle threshold (Ct) for detecting bacterial DNA was significantly lower in the Eswabs, indicating a higher amount of bacterial DNA in these swabs (Supplementary Figure 1). However, despite this higher bacterial DNA, the number of observed OTUs was lower in Eswabs than in cotton swabs (Supplementary Figure 2). The overall bacterial composition in the buccal swabs was mostly determined by the individual from which the sample came from and less by the swab type. The Bray-Curtis based PCoA shows clearly that samples from the same individual cluster together, while no clustering based on swab type was visible (Supplementary Figure 3). Indeed, 71% of the variation between samples was explained by the individual from which samples was taken (Adonis, R^2 0.714, $p < 0.001$ using 999 permutations), while the type of swab did not significantly affect the variation between samples (Adonis, R^2 0.048, $p = 0.513$ using 999 permutations). Furthermore, the pattern of relative abundances at phylum and genus level showed a more clear pattern for individuality than for swab type (Supplementary Figures 4 and 5). These results indicate that

the overall bacterial community composition of the buccal mucosa was not significantly affected by swab type and preservation fluid, although richness (number of observed OTUs) was lower in Eswabs compared with cotton swabs.

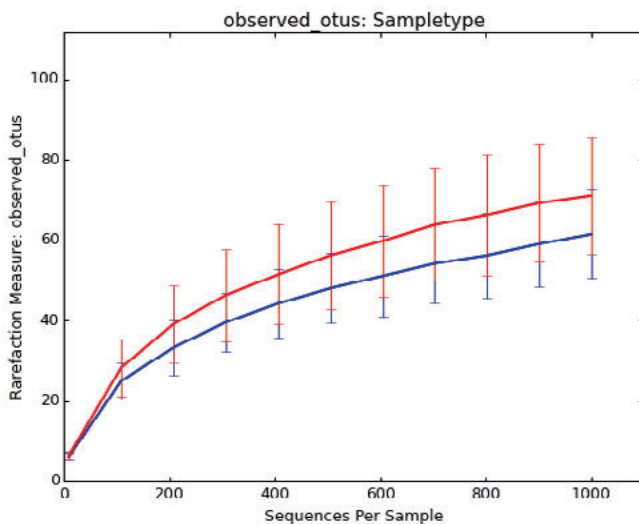
SUPPLEMENTARY FIGURES



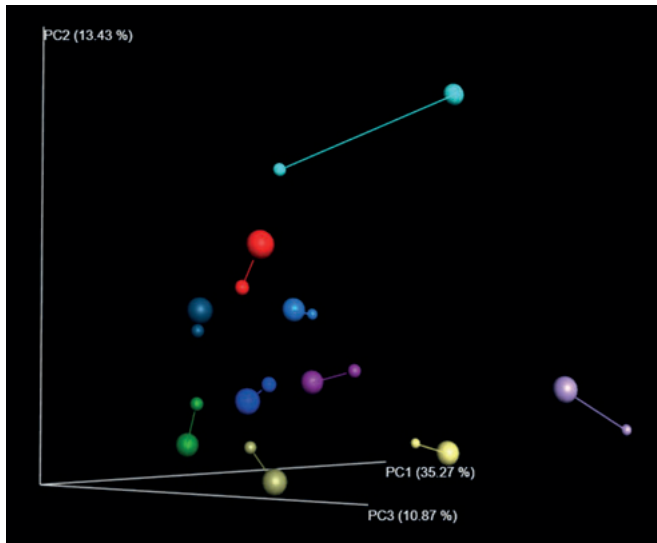
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SUPPLEMENTARY FIGURE S1: Threshold cycles and DNA concentration in two swab types.

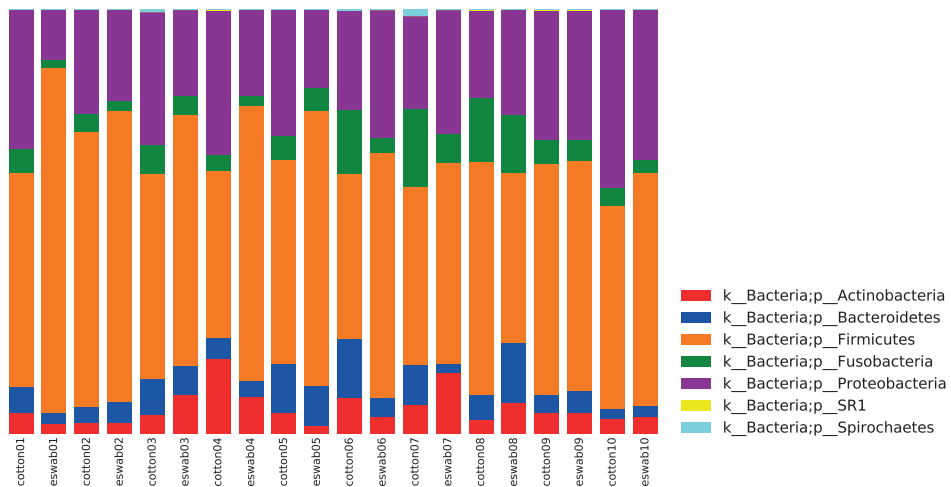
Significantly less threshold cycles (Ct) were needed to detect a signal of the targeted bacterial nucleic acid in Eswabs compared with cotton swabs ($p=0.002$, paired t-test), indicating that more bacterial DNA was present in Eswabs from the dry mouth patient cohort. No significant difference was found between the two sample types in total DNA concentration (ng/μl).



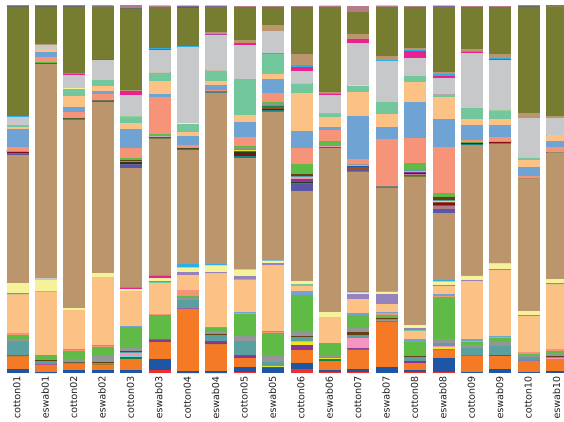
SUPPLEMENTARY FIGURE S2: Alpha-rarefaction plot of two swab types. Red curve = cotton swabs, blue curve = Eswabs. Sequence depth was cut-of at 1000 reads/sample. Higher number of different OTUs observed in cotton swabs than Eswabs at same sequencing depth.



SUPPLEMENTARY FIGURE S3: PCoA of two swab types in 10 healthy volunteers . PCoA based on Bray-Curtis distance matrix, explaining 59% of the variation within the first three principal coordinates (PC1-PC3). Dots are coloured according to subject. Large dots are cotton swabs, small dots Eswabs. A vector line is drawn between the two sample types of the same individual.

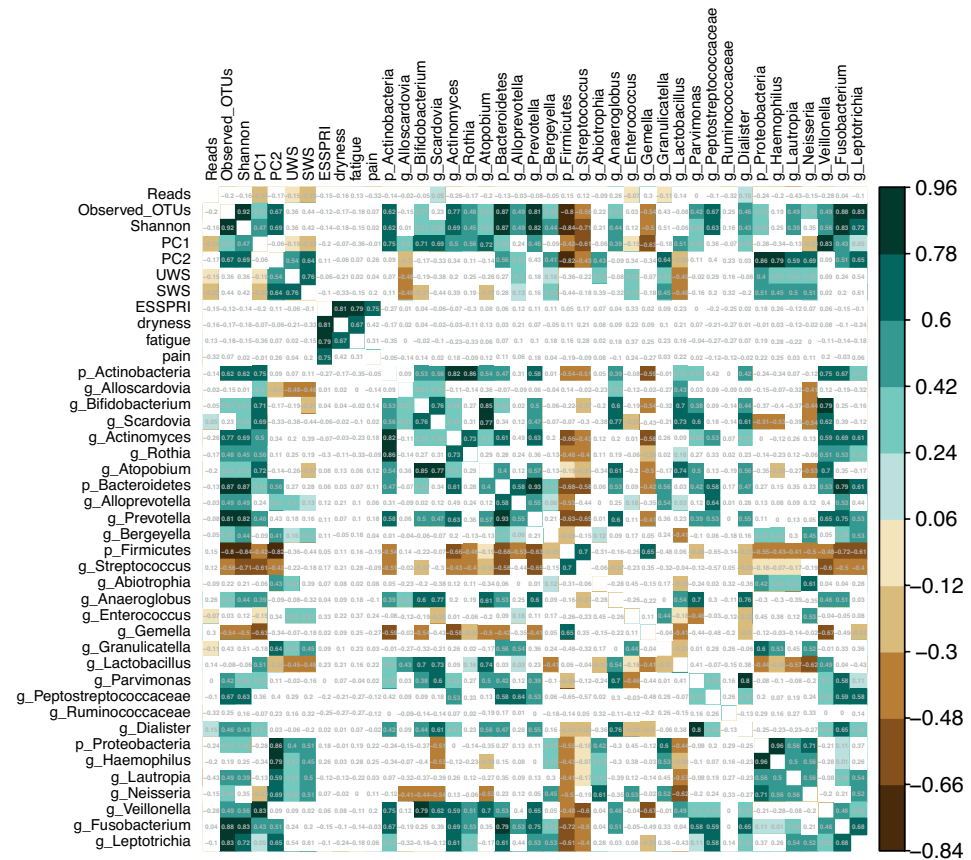


SUPPLEMENTARY FIGURE S4: Relative abundance of bacterial taxa at phylum level per buccal swab sample. Samples are ordered on subject, with cotton and eswab placed side by side (x-axis) and relative abundance as y-axis.



SUPPLEMENTARY FIGURE S5: Relative abundance of bacterial taxa at genus level per buccal swab type. Samples are ordered by subject, with cotton and eswab placed side by side (x-axis) and relative abundance as y-axis. Legend is shown below.

- k_Bacteria;p_Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Actinomycetaceae;g_Actinobaculum
- k_Bacteria;p_Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Actinomycetaceae;g_Actinomyces
- k_Bacteria;p_Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Micrococcaceae;g_Rothia
- k_Bacteria;p_Actinobacteria;c_Actinobacteria;o_Bifidobacteriales;f_Bifidobacteriaceae;g_Scardovia
- k_Bacteria;p_Actinobacteria;c_Actinobacteria;o_Corynebacteriales;f_Corynebacteriaceae;g_Corynebacterium
- k_Bacteria;p_Actinobacteria;c_Coriobacteriales;f_Coriobacteriaceae;g_Atopobium
- k_Bacteria;p_Bacteroidetes;c_Bacteroidetes_[C-1];o_Bacteroidetes_[O-1];f_Bacteroidetes_[F-1];g_Bacteroidetes_[G-5]
- k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Bacteroidales_[F-2];g_Bacteroidales_[G-2]
- k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Porphyrionadaceae;g_Porphyrionas
- k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Porphyrionadaceae;g_Tannerella
- k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Prevotellaceae;g_Alloprevotella
- k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Prevotellaceae;g_Prevotella
- k_Bacteria;p_Bacteroidetes;c_Flavobacteriales;f_Flavobacteriaceae;g_Bergeyella
- k_Bacteria;p_Bacteroidetes;c_Flavobacteriales;f_Flavobacteriaceae;g_Capnocytophaga
- k_Bacteria;p_Firmicutes;c_Bacilli;o_Bacillales;f_Gemellaceae;g_Gemella
- k_Bacteria;p_Firmicutes;c_Bacilli;o_Bacillales;f_Staphylococcaceae;g_Staphylococcus
- k_Bacteria;p_Firmicutes;c_Bacilli;o_Lactobacillales;f_Aerococcaceae;g_Abiotrophia
- k_Bacteria;p_Firmicutes;c_Bacilli;o_Lactobacillales;f_Carnobacteriaceae;g_Granulicatella
- k_Bacteria;p_Firmicutes;c_Bacilli;o_Lactobacillales;f_Enterococcaceae;g_Enterococcus
- k_Bacteria;p_Firmicutes;c_Bacilli;o_Lactobacillales;f_Lactobacillaceae;g_Lactobacillus
- k_Bacteria;p_Firmicutes;c_Bacilli;o_Lactobacillales;f_Streptococcaceae;g_Lactococcus
- k_Bacteria;p_Firmicutes;c_Bacilli;o_Lactobacillales;f_Streptococcaceae;g_Streptococcus
- k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae_[XIV];g_Catonella
- k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae_[XIV];g_Lachnoanaerobaculum
- k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae_[XIV];g_Lachnospiraceae_[G-2]
- k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae_[XIV];g_Lachnospiraceae_[G-3]
- k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae_[XIV];g_Oribacterium
- k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae_[XIV];g_Stomatobaculum
- k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Peptoniphilaceae;g_Parvimonas
- k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Peptostreptococcaceae_[XI];g_Mogibacterium
- k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Peptostreptococcaceae_[XI];g_Peptostreptococcaceae_[XII][G-7]
- k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Peptostreptococcaceae_[XI];g_Peptostreptococcaceae_[XII][G-9]
- k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Peptostreptococcaceae_[XI];g_Peptostreptococcus
- k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Ruminococcaceae_[G-1]
- k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Ruminococcaceae_[G-2]
- k_Bacteria;p_Firmicutes;c_Erysipelotrichia;o_Erysipelotrichales;f_Erysipelotrichaceae;g_Solobacterium
- k_Bacteria;p_Firmicutes;c_Negativicutes;o_Selenomonadales;f_Veillonellaceae;g_Anaeroglobus
- k_Bacteria;p_Firmicutes;c_Negativicutes;o_Selenomonadales;f_Veillonellaceae;g_Dialister
- k_Bacteria;p_Firmicutes;c_Negativicutes;o_Selenomonadales;f_Veillonellaceae;g_Megasphaera
- k_Bacteria;p_Firmicutes;c_Negativicutes;o_Selenomonadales;f_Veillonellaceae;g_Mitsuokella
- k_Bacteria;p_Firmicutes;c_Negativicutes;o_Selenomonadales;f_Veillonellaceae;g_Selenomonas
- k_Bacteria;p_Firmicutes;c_Negativicutes;o_Selenomonadales;f_Veillonellaceae;g_Veillonella
- k_Bacteria;p_Fusobacteria;c_Fusobacteriales;f_Fusobacteriaceae;g_Fusobacterium
- k_Bacteria;p_Fusobacteria;c_Fusobacteriales;f_Leptotrichiaceae;g_Leptotrichia
- k_Bacteria;p_Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Burkholderiaceae;g_Lautropia
- k_Bacteria;p_Proteobacteria;c_Betaproteobacteria;o_Neisseriales;f_Neisseriaceae;g_Eikenella
- k_Bacteria;p_Proteobacteria;c_Betaproteobacteria;o_Neisseriales;f_Neisseriaceae;g_Kingella
- k_Bacteria;p_Proteobacteria;c_Betaproteobacteria;o_Neisseriales;f_Neisseriaceae;g_Neisseria
- k_Bacteria;p_Proteobacteria;c_Epsilonproteobacteria;o_Campylobacteriales;f_Campylobacteriaceae;g_Campylobacter
- k_Bacteria;p_Proteobacteria;c_Gammaproteobacteria;o_Cardiobacteriales;f_Cardiobacteriaceae;g_Cardiobacterium
- k_Bacteria;p_Proteobacteria;c_Gammaproteobacteria;o_Pasteurellales;f_Pasteurellaceae;g_Aggregatibacter
- k_Bacteria;p_Proteobacteria;c_Gammaproteobacteria;o_Pasteurellales;f_Pasteurellaceae;g_Haemophilus
- k_Bacteria;p_SR1;c_SR1_[C-1];o_SR1_[O-1];f_SR1_[F-1];g_SR1_[G-1]
- k_Bacteria;p_Spirochaetes;c_Spirochaetiales;f_Spirochaetaceae;g_Treponema



SUPPLEMENTARY FIGURE S6: Correlation plot of clinical and bacterial data. All coloured squares indicate a significant Spearman correlation ($q < 0.01$). White squares are not significantly correlated. The colour indicates the direction (green = positive, brown = negative) of the Spearman's rho correlation. Exact Spearman's rho values are printed in the squares.

SUPPLEMENTARY TABLES

SUPPLEMENTARY TABLE S1: Associations of bacterial taxa with pSS and non-SS sicca^a

Taxon	Groups compared	Coefficient	N	N not 0	P-value	Q-value
p.Firmicutes_c.Bacilli_o.Lactobacillales_f.Carnobacteriaceae	pSS vs HC	-0.05	61	53	0.0001	0.0129
p.Firmicutes_c.Bacilli_o.Lactobacillales_f.Carnobacteriaceae_g.Granulicatella	pSS vs HC	-0.05	61	53	0.0001	0.0129
p.Firmicutes_c.Bacilli_o.Lactobacillales_f.Aerococcaceae	pSS vs HC	-0.02	61	21	0.0008	0.0569
p.Firmicutes_c.Bacilli_o.Lactobacillales_f.Aerococcaceae_g.Abiotrophia	pSS vs HC	-0.02	61	21	0.0008	0.0569
p.Proteobacteria_c.Betaproteobacteria_o.Burkholderiales	pSS vs HC	-0.05	61	35	0.0043	0.0803
p.Proteobacteria_c.Betaproteobacteria_o.Burkholderiales_f.Burkholderiaceae	pSS vs HC	-0.05	61	35	0.0043	0.0803
p.Proteobacteria_c.Betaproteobacteria_o.Burkholderiales_f.Burkholderiaceae_g.Lautropia	pSS vs HC	-0.05	61	35	0.0043	0.0803
p.Bacteroidetes_c.Flavobacteriia_o.Flavobacteriales_f.Flavobacteriaceae_g.Bergeyella	pSS vs HC	-0.02	61	37	0.0031	0.0803
p.Firmicutes_c.Bacilli_o.Lactobacillales_f.Enterococcaceae	pSS vs HC	-0.02	61	29	0.0030	0.0803
p.Firmicutes_c.Bacilli_o.Lactobacillales_f.Enterococcaceae_g.Enterococcus	pSS vs HC	-0.02	61	29	0.0030	0.0803
p.Firmicutes_c.Clostridia_o.Clostridiales_f.Ruminococcaceae	pSS vs HC	-0.01	61	14	0.0021	0.0803
p.Actinobacteria_c.Actinobacteria_o.Bifidobacteriales	pSS vs HC	0.03	61	21	0.0029	0.0803
p.Actinobacteria_c.Actinobacteria_o.Bifidobacteriales_f.Bifidobacteriaceae	pSS vs HC	0.03	61	21	0.0029	0.0803
p.Proteobacteria	pSS vs HC	-0.16	61	58	0.0058	0.0821
p.Proteobacteria_c.Betaproteobacteria	pSS vs HC	-0.10	61	48	0.0050	0.0821
p.Proteobacteria_c.Betaproteobacteria_o.Neisseriales_f.Neisseriaceae_g.Neisseria	pSS vs HC	-0.09	61	46	0.0060	0.0821
p.Actinobacteria_c.Actinobacteria_o.Bifidobacteriales_f.Bifidobacteriaceae_g.Alloscardovia	pSS vs HC	0.01	61	7	0.0057	0.0821
p.Firmicutes_c.Negativicutes_o.Selenomonadales_f.Veillonellaceae_g.Anaeroglobus	pSS vs HC	0.02	61	9	0.0050	0.0821
p.Proteobacteria_c.Betaproteobacteria_o.Neisseriales	pSS vs HC	-0.09	61	48	0.0078	0.0890
p.Proteobacteria_c.Betaproteobacteria_o.Neisseriales_f.Neisseriaceae	pSS vs HC	-0.09	61	48	0.0078	0.0890
p.Actinobacteria_c.Actinobacteria_o.Bifidobacteriales_f.Bifidobacteriaceae_g.Scardovia	pSS vs HC	0.03	61	15	0.0081	0.0890
p.Firmicutes_c.Clostridia_o.Clostridiales_f.Peptoniphilaceae	pSS vs HC	0.02	61	20	0.0095	0.0931
p.Firmicutes_c.Clostridia_o.Clostridiales_f.Peptoniphilaceae_g.Parvimonas	pSS vs HC	0.02	61	20	0.0095	0.0931
p.Firmicutes_c.Clostridia_o.Clostridiales_f.Ruminococcaceae_g.Ruminococcaceae	pSS vs HC	-0.01	61	7	0.0104	0.0940

SUPPLEMENTARY TABLE S1: (Continued)

Taxon	Groups compared	Coefficient	N	N not 0	P-value	Q-value
p.Actinobacteria_c.Actinobacteria_o.Bifidobacteriales_f. Bifidobacteriaceae_g.Bifidobacterium	pSS vs HC	0.03	61	14	0.0125	0.1031
p.Proteobacteria_c.Gammaproteobacteria_o.Pasteurellales	pSS vs HC	-0.13	61	58	0.0161	0.1144
p.Proteobacteria_c.Gammaproteobacteria_o.Pasteurellales_f. Pasteurellaceae	pSS vs HC	-0.13	61	58	0.0161	0.1144
p.Proteobacteria_c.Gammaproteobacteria	pSS vs HC	-0.13	61	58	0.0178	0.1144
p.Proteobacteria_c.Gammaproteobacteria_o.Pasteurellales_f. Pasteurellaceae_g.Haemophilus	pSS vs HC	-0.13	61	58	0.0195	0.1189
p.Actinobacteria_c.Coriobacteriia	pSS vs HC	0.02	61	31	0.0211	0.1189
p.Actinobacteria_c.Coriobacteriia_o.Coriobacteriales	pSS vs HC	0.02	61	31	0.0211	0.1189
p.Actinobacteria_c.Coriobacteriia_o.Coriobacteriales_f. Coriobacteriaceae	pSS vs HC	0.02	61	31	0.0211	0.1189
p.Actinobacteria_c.Coriobacteriia_o.Coriobacteriales_f. Coriobacteriaceae_g.Atopobium	pSS vs HC	0.02	61	31	0.0211	0.1189
p.Firmicutes_c.Clostridia_o.Clostridiales_f.Peptostreptococcaceae_ Xlg.Peptostreptococcaceae	pSS vs HC	0.01	61	11	0.0216	0.1190
p.Bacteroidetes_c.Bacteroidia_o.Bacteroidales_f.Prevotellaceae_g. Alloprevotella	pSS vs HC	-0.03	61	40	0.0240	0.1240
p.Firmicutes_c.Negativicutes_o.Selenomonadales_f. Veillonellaceae_g.Dialister	pSS vs HC	0.01	61	16	0.0273	0.1393
p.Firmicutes_c.Bacilli_o.Lactobacillales_f.Lactobacillaceae	pSS vs HC	0.01	61	20	0.0321	0.1592
p.Firmicutes_c.Bacilli_o.Lactobacillales_f.Lactobacillaceae_g. Lactobacillus	pSS vs HC	0.01	61	20	0.0321	0.1592
p.Firmicutes_c.Bacilli_o.Lactobacillales_f.Carnobacteriaceae	pSS vs non-SS	-0.03	123	108	0.0039	0.0997
p.Firmicutes_c.Bacilli_o.Lactobacillales_f.Carnobacteriaceae_g. Granulicatella	pSS vs non-SS	-0.03	123	108	0.0039	0.0997
p.Actinobacteria_c.Actinobacteria_o.Bifidobacteriales	pSS vs non-SS	0.02	123	45	0.0096	0.1213
p.Actinobacteria_c.Actinobacteria_o.Bifidobacteriales_f. Bifidobacteriaceae	pSS vs non-SS	0.02	123	45	0.0096	0.1213
p.Bacteroidetes_c.Flavobacteriia_o.Flavobacteriales_f. Flavobacteriaceae_g.Bergeyella	pSS vs non-SS	-0.01	123	75	0.0161	0.1513
p.Bacteroidetes_c.Bacteroidia_o.Bacteroidales_f.Prevotellaceae_g. Alloprevotella	Non-SS vs HC	-0.03	110	66	0.0014	0.0446
p.Firmicutes_c.Clostridia_o.Clostridiales_f.Ruminococcaceae_g. Ruminococcaceae_G2	Non-SS vs HC	-0.01	110	15	0.0232	0.1738
p.Firmicutes_c.Clostridia_o.Clostridiales_f.Ruminococcaceae	Non-SS vs HC	-0.01	110	27	0.0290	0.1880
p.Firmicutes_c.Bacilli_o.Lactobacillales_f.Carnobacteriaceae_g. Granulicatella	pSS vs pop. control	-0.07	140	129	0.0000	0.0000

SUPPLEMENTARY TABLE S1: (Continued)

Taxon	Groups compared	Coeffi- cient	N		P-value	Q-value
			N	not 0		
p.Firmicutes_c.Bacilli_o.Lactobacillales_f.Aerococcaceae_g. Abiotrophia	pSS vs pop. control	-0.02	140	67	0.0016	0.0565
p.Firmicutes_c.Clostridia_o.Clostridiales_f.Ruminococcaceae_g. Ruminococcaceae_G1	pSS vs pop. control	-0.01	140	40	0.0050	0.1003
p.Proteobacteria_c.Betaproteobacteria_o.Burkholderiales_f. Burkholderiaceae_g.Lautropia	pSS vs pop. control	-0.04	140	87	0.0056	0.1003
p.Firmicutes_c.Bacilli_o.Lactobacillales_f.Carnobacteriaceae_g. Granulicatella	Non-SS vs pop. control	-0.04	189	182	0.0000	0.0009
p.Firmicutes_c.Clostridia_o.Clostridiales_f.Ruminococcaceae_g. Ruminococcaceae_G1	Non-SS vs pop. control	-0.01	189	48	0.0001	0.0028
p.Firmicutes_c.Bacilli_o.Lactobacillales_f.Enterococcaceae_g. Enterococcus	Non-SS vs pop. control	0.01	189	67	0.0006	0.0140
p.Firmicutes_c.Bacilli_o.Lactobacillales_f.Aerococcaceae_g. Abiotrophia	Non-SS vs pop. control	-0.02	189	91	0.0033	0.0449
p.Proteobacteria_c.Betaproteobacteria_o.Burkholderiales_f. Burkholderiaceae_g.Lautropia	Non-SS vs pop. control	-0.02	189	120	0.0061	0.0687
p.Firmicutes_c.Bacilli_o.Lactobacillales_f.Lactobacillaceae_g. Lactobacillus	Non-SS vs pop. control	-0.01	189	74	0.0095	0.0927

^aAnalysis performed in MaAsLin, taking into account smoking and dental status. Sex was taken into account for the analysis of associations between pSS and non-SS sicca (non-SS) compared with population controls. pSS; primary Sjögren's syndrome patients; non-SS sicca: non-Sjögren's syndrome patients; HCs: healthy controls; pop.control: population controls; p.: phylum; c.: class; o.: order; f.: family; g.: genus. Coefficient shows the difference in arcsin square-root relative abundance between groups. Positive is higher in first named group in column 2; negative means lower in first named group; N: total number of samples imported in the analysis; N not 0: number of samples where the relative abundance of that taxon was higher than 0. Q-value is the false discovery corrected p-value.

SUPPLEMENTARY TABLE S2: Associations of bacterial taxa with pSS taking SWS secretin rate into account^a

Taxon	Groups compared	Coefficient	N	N not 0	P-value	Q-value
p.Firmicutes_c.Bacilli_o.Lactobacillales_f.Carnobacteriaceae_g.Granulicatella	pSS vs HC	-0.05	61	53	0.0000	0.0080
p.Firmicutes_c.Bacilli_o.Lactobacillales_f.Carnobacteriaceae	pSS vs HC	-0.05	61	53	0.0000	0.0080
p.Firmicutes_c.Bacilli_o.Lactobacillales_f.Aerococcaceae	pSS vs HC	-0.02	61	21	0.0004	0.0304
p.Firmicutes_c.Bacilli_o.Lactobacillales_f.Aerococcaceae_g.Abiotrophia	pSS vs HC	-0.02	61	21	0.0004	0.0304
p.Proteobacteria	pSS vs HC	-0.17	61	58	0.0013	0.0613
p.Firmicutes_c.Clostridia_o.Clostridiales_f.Ruminococcaceae	pSS vs HC	-0.01	61	14	0.0020	0.0684
p.Firmicutes_c.Bacilli_o.Lactobacillales_f.Enterococcaceae	pSS vs HC	-0.02	61	29	0.0027	0.0740
p.Firmicutes_c.Bacilli_o.Lactobacillales_f.Enterococcaceae_g.Enterococcus	pSS vs HC	-0.02	61	29	0.0027	0.0740
p.Firmicutes_o.Negativicutes_o.Selenomonadales_f.Veillonellaceae_g.Anaeroglobus	pSS vs HC	0.02	61	9	0.0033	0.0786
p.Actinobacteria_c.Actinobacteria_o.Bifidobacteriales_f.Bifidobacteriaceae_g.Scardovia	pSS vs HC	0.03	61	15	0.0033	0.0786
p.Bacteroidetes_c.Flavobacteriia_o.Flavobacteriales_f.Flavobacteriaceae_g.Bergeyella	pSS vs HC	-0.02	61	37	0.0034	0.0786
p.Actinobacteria_c.Actinobacteria_o.Bifidobacteriales_f.Bifidobacteriaceae_g.Bifidobacterium	pSS vs HC	0.03	61	14	0.0071	0.1070
p.Firmicutes_c.Clostridia_o.Clostridiales_f.Ruminococcaceae_g.Ruminococcaceae_G1	pSS vs HC	-0.01	61	7	0.0090	0.1268
p.Firmicutes_c.Clostridia_o.Clostridiales_f.Peptoniphilaceae	pSS vs HC	0.02	61	20	0.0103	0.1332
p.Firmicutes_c.Clostridia_o.Clostridiales_f.Peptoniphilaceae_g.Parvimonas	pSS vs HC	0.02	61	20	0.0103	0.1332

^aAnalysis performed in MaAsLin, taking into account smoking and dental status and SWS secretion rate. pSS: primary Sjögren's syndrome patients, non-SS sicca: non-Sjögren's syndrome patients, HCs: healthy controls, pop.control: population controls. p.: phylum, c.: class, o.: order, f.: family, g.: genus. Coefficient shows the difference in arcsin square-root relative abundance between groups. Positive is higher in first named group in column 2, negative means lower in first named group. N: total number of samples imported in the analysis. N not 0: number of samples where the relative abundance of that taxon was higher than 0. Q-value is the false discovery corrected p-value.

