UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE RIBEIRÃO PRETO

DAIRA ESTER DE SOUSA

Análise da microbiota oral e associação com a condição bucal em pacientes com Síndrome de Sjögren

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Trabalho de Conclusão de Curso apresentado à Faculdade Odontologia de Ribeirão Preto da Universidade de São Paulo, como parte dos requisitos para obtenção do grau de Cirurgião(ã)-Dentista.

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RESUMO

SOUSA, Daira Ester de. **Análise da condição bucal e microbiota oral de pacientes com Síndrome de Sjögren**. 2023. 25 f. Trabalho de Conclusão de Curso – Faculdade de Odontologia de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, 2023.

Objetivo: Síndrome de Sjögren (SS) é uma doença sistêmica autoimune, caracterizada pela infiltração linfocitária no tecido glandular, de evolução crônica, acometendo principalmente as glândulas exócrinas salivares e lacrimais. Considerando o impacto da doença na cavidade oral devido à hipossalivação, o objetivo desse trabalho foi avaliar a população microbiana oral em pacientes com SS, e correlacioná-las com as variáveis clínicas: xerostomia, hipossalivação, condição dental (CPOD), periodontal (PSR) e ocorrência de candidíase oral.

Métodos: Trata-se de estudo transversal em que foram analisados dados e amostras de pacientes distribuídos em dois grupos de estudo, pareados por sexo e idade: 35 pacientes com diagnóstico de SS (grupo SS) e 35 amostras do grupo controle (sem diagnóstico de SS). Todos os pacientes foram submetidos à anamnese e exame físico intraoral. O exame físico incluiu: Fluxo salivar não estimulado (FSNE), índice de placa (IP), exame periodontal simplificado (PSR), índice CPOD (dentes cariados, perdidos e obturados), e uso de prótese dentária. A ocorrência de candidíase oral foi avaliada por contagem de unidades formadoras de colônias (UFC) e ágar cromogênico (CHROMagar).

Resultados: Na análise da diversidade microbiana por abundância relativa, houve uma diferença significativa entre os pacientes do grupo SS e o grupo controle, sendo o filo *Firmicutes* mais abundante no grupo SS e os filos *Actinobacteriota, Bacteroidota e Fusobacteriota* mais abundante no grupo controle. Quanto às análises clínicas, em relação ao PSR pacientes do grupo SS apresentaram menor diversidade bacteriana, mas bolsas periodontais maiores que 3,5mm foram mais prevalentes.

Conclusão: Diferenças entre a microbiota total da saliva foram identificadas no grupo SS em comparação com o grupo controle. Uma microbiota menos diversa foi encontrada em pacientes com SS, em associação com pontuações mais altas em índices clínicos como CPOD, PSR e IP e independente do escore de fluxo salivar.

Palavras-chaves. Síndrome de Sjögren. Hipossalivação. Microbiota oral. Complicações orais.

ABSTRACT

SOUSA, Daira Ester de. **Analysis of the oral condition and oral microbiota of patients with Sjögren's Syndrome**. 2023. 25 f. Completion of course work – Faculty of Dentistry of Ribeirão Preto, University of São Paulo, Ribeirão Preto, 2023.

Objective: Sjögren's Syndrome (SS) is a systemic autoimmune disease, characterized by lymphocytic infiltration in the glandular tissue, of chronic evolution, affecting mainly the exocrine salivary and lacrimal glands. Considering the impact of the disease on the oral cavity due to hyposalivation, the objective of this work is to evaluate the oral microbial population in patients with Sjögren's Syndrome, and to correlate them with the clinical variables: xerostomia, hyposalivation, dental condition (DMFT), periodontal disease (PSR) and occurrence of oral candidiasis.

Methods: The exception of 35 patients diagnosed with Sjögren's syndrome (SS group) and 35 samples from the control group (without SS diagnosis) matched by sex and age were pregnant women. All patients were admitted for anamnesis and intraoral clinical examination. The intraoral clinical examination included: Unstimulated salivary flow (FSNE), Plaque index (PI), Simplified periodontal examination (PSR), CPOD index (decayed, missing and filled teeth), Use of dental prosthesis. Occurrence of oral candidiasis was evaluated by counting colony forming units (CFU) and chromogenic agar (CHROMagar).

Results: In the analysis of microbial diversity by relative abundance, there was a significant difference between patients in the SS group and the control group, with the *Firmicutes* phylum being more abundant in the SS group and the *Actinobacteriota*, *Bacteroidota* and *Fusobacteriota* phyla more abundant in the control group. As for the clinical analyses, in relation to PSR, patients in the SS group had less bacterial diversity, but periodontal pockets larger than 3.5 mm were more prevalent.

Conclusion: Differences between the total salivary microbiota were identified in the SS group compared to the control group. A less diverse microbiota was found in patients with SS, in association with higher scores on clinical indices such as CPOD, PSR and PI and independent of salivary flow score.

Keywords: Sjögren's Syndrome. Hyposalivation. Oral microbiota. Oral complications.

SUMÁRIO

1 – Introduction

Sjögren's Syndrome (SS) is a systemic autoimmune disease, characterized by lymphocytic infiltration in the glandular tissue, of chronic evolution, affecting mainly affects the exocrine salivary and lacrimal glands. SS can be classified as primary SS, when it affects the salivary and lacrimal glands, causing dry eyes and mouth, in an isolated form, that is, without the presence of another autoimmune disease; and as secondary SS, when it is associated with another rheumatic disease, such as systemic lupus erythematosus, rheumatoid arthritis, systemic sclerosis (37).

SS is an autoimmune disease, which affects 0.5-3% of the adult population, especially middle-aged women, with a male/female ratio of 1:9 (1). The systemic involvement of the syndrome stimulates different clinical manifestations and, occasionally, extraglandular manifestations may occur during the initial stages of the disease. Although the pathogenesis of the dSS has not yet been fully elucidated, substantial data demonstrate that SS is an autoimmune disease with anti-SSa/Ro and anti-SSb/La autoantibodies acting as immunological markers (2).

In the oral cavity, the most common clinical manifestations have as primary cause the hypofunction of the salivary glands and consequent hyposalivation or low levels of saliva secretion, including atrophy of the lingual papillae, fissured tongue and, erythema and pain of the oral mucosa. Hyposalivation leads to xerostomia, a subjective feeling of dry mouth, which has a negative impact on the quality of life of patients, since it leads to difficulty in swallowing, speaking, and changes in taste. In addition to these symptoms, caries, periodontal disease and fungal infections such as candidiasis are considered the main oral complications secondary to hyposalivation. Even though the predisposition to oral diseases is known, the evidence of the association between SS and these complications is conflicting, especially in relation to periodontal disease and candidiasis.

Several clinical studies have shown a greater number of decayed teeth and a higher frequency of periodontal disease and candidiasis in individuals with SS compared to healthy individuals (3,4), however other studies report that there are no differences in the frequency of these alterations in patients with SS (5,6).

Studies on the microbiota of SS patients revealed increased levels of *Streptococcus mutans*, L*actobacillus* spp. and *Candida albicans* in supragingival biofilm samples (7). Samples from the oral mucosa and tongue also showed a higher prevalence of *Staphylococcus aureus* and *C. albicans*. Still, the reduction of salivary flow and the maintenance of hyposalivation increase the susceptibility to bacterial and fungal diseases (8). Recent studies

indicate that alterations in the oral microbiota are associated with hyposalivation, as well as an association with high prevalence of candidiasis in SS patients, meaning that SS could be considered as an independent predictor for the onset of candidiasis (9). Organisms such as *Capnocytophaga*, *Dialister*, *Fusobacterium*, *Helicobacter*, *Streptococcus* and *Veilonella* were found in abundance in SS, whereas *Porphyromonas* gingivalis and *Actinobacillus actinomycetemcomitans* were not isolated in SS patients (10).

Other studies have indicated that changes in the oral microbiota may be present in SS, regardless of hyposalivation effects, as reported by Huma et al. 2016, where patients with pSS presented dysbiosis of the oral microbiota of the total saliva when compared to controls showing that the displacement of the oral microbiota of patients with pSS can occur independently of hyposalivation (11,12). On the other hand, other studies evaluated that the total count of bacteria in saliva is similar among patients with SS, but the decrease in salivary flow is related to an increase in the concentration of some microorganisms, such as *Lactobacillus acidophilus*, *S. mutans* and *C. albicans*, which in turn may be associated with a higher rate of caries and candidiasis in these patients (13).

Faced with conflicting findings in the literature regarding oral health conditions and also the composition of the oral microbiota of patients with SS (15), this study aims to determine the oral microbial population, as well as the oral condition of patients with SS. In addition, we compare the oral microbiological population of patients with SS with that of healthy patients and correlate microbiological findings with salivary flow, bacterial plaque index, dental condition (CPOD index), periodontal condition (PSR) and occurrence of oral candidiasis.

2 – Materials and Methods

2.1 - Samples and Study Design

This is a cross-sectional study in which samples from patients divided into two study groups, matched by sex and age, were analyzed: 35 patients diagnosed with SS (SS group) and 35 patients in the control group (without SS diagnosis). The project was submitted and approved by the Research Ethics Committee of the School of Dentistry of Ribeirão Preto – University of São Paulo (FORP-USP) (CAAE: 95703618.4.0000.5419) (APPENDIX). All patients were informed about the research objectives and signed an informed consent form. Patients in the SS group were recruited from among patients being treated at the Rheumatology Division of the Hospital das Clínicas, Ribeirão Preto Medical School – USP or being treated at the FORP-USP, for treatment of SS, during the period from October 2018 to

September 2019. Patients in the control group were recruited from among individuals referred to FORP – USP by Basic Health Units in the Ribeirão Preto region, for routine dental treatment, from October 2018 to October 2022.

Inclusion criteria for the SS group consisted of diagnosis of SS according to the criteria of the American-European Consensus Group (20). Exclusion criteria for both groups were patients with a history of head and neck radiotherapy, history of neoplasms or salivary gland infections, acquired immunodeficiency virus infection, sarcoidosis, viral hepatitis, diabetes mellitus, and active smokers.

All patients underwent anamnesis and intraoral physical examination. The intraoral clinical examination included:

- Non-stimulated salivary flow (NSSF): participants were instructed not to eat, drink, or brush their teeth for at least 1 hour prior to collection, in order to minimize the risk of contamination. The evaluation of salivary flow was determined by expectoration of saliva in a 15mL graduated tube for a period of 15 minutes. The volume obtained was calculated per minute.
- Plaque index (modification of the Silness and Loe 1964 index): the presence of biofilm in the gingival area of the teeth was determined, based on its thickness, classified in 4 grades: Grade 0 – No film on the gingival area of the tooth surface; Grade 1 – Film of plaque adhered to the gingival margin and the area adjacent to the tooth seen only when removed by the probe; Grade 2 – Film of medium thickness covering the gingival area, visible to the naked eye; Grade 3 - Very thick film on the entire face of the tooth.
- Periodontal Screening and Recording (PSR): the mouth was divided into sextants, six sites per tooth were probed and codes from 0 to 4 were recorded. 0 = Absence of clinical signs; 1 = Bleeding on probing; 2 = Presence of supra and/or subgingival calculus and/or defective restorative margins; 3 = Presence of periodontal pocket of 3.5 to 5.5mm; and 4 = Presence of a periodontal pocket greater than 5.5mm.
- DMFT Index (Decayed, Missing, and Filled Teeth): the evaluation followed the severity scale established by the WHO, with a very low prevalence (0.1-1.1); low prevalence (1.2-2.6); moderate prevalence (2.7-4.4); and high prevalence (4.5-6.5).
- Use of dental prosthesis: Such as Fixed Partial Dentures (FPD), Removable Partial Dentures (RPD) and Complete Dentures (CD).

The occurrence of oral candidiasis was evaluated by counting colony forming units (CFU) and chromogenic agar (CHROMagar). Focus Score (FS) of SS patients was assessed through medical records.

2.2 - Sample collection

Samples of whole saliva were collected using a mouthwash with sterile phosphate buffer solution (PBS) pH 7.3, 0.1M, using the technique described by Samaranayake (18), in 1986. Patients were invited to rinse their mouth with 10ml of PBS for 60 seconds and then expectorate into a falcon tube with the aid of a funnel. The falcon tube containing the rinse was subsequently centrifuged (2000g for 10 minutes), the supernatant was discarded, and the solid residue deposited at the bottom of the falcon tube resuspended in sterile PBS until reaching 1ml of solution. From that 1ml of solution obtained, divided into two portions, were obtained a sample for culture of *Candida* spp, and another sample for extraction of bacterial genomic DNA.

2.3 - Characterization of the oral microbiota using 16S rRNA gene amplification and sequencing

Genomic DNA was extracted from each sample using the technique of Doyle & Doyle 1990 modified with CTAB (38,39). DNA purity was assessed using UV/Visible Spectrophotometer. Extracted genomic DNA samples were stored at -80°C until use. The extracted samples of genomic DNA were subjected to PCR amplification of the 16S ribosomal gene (V3-V4 regions), using the QIAseq 16S/ITS Panel kit (Qiagen). The PCR reaction was performed in a total volume of 10 µl, containing: microbial genomic DNA (1 µl); mixture of forward and reverse primers specific for the V3-V4 regions of 16S (1 µl); master mix from the QIAseq 16S/ITS Panel Kit, containing reagents (2.5 µl) and nuclease-free water (5.5 µl). The PCR was performed in an Eppendorf thermal cycler, model Mastercycler epgradientS, with the following conditions: 95°C for 2 minutes; 20 cycles of 95°C for 30 seconds, 50°C for 30 seconds, 72°C for 2 minutes; final extension at 72°C for 5 minutes, and keeping the sample at 4°C until the moment of purification.

After amplification, 20 µl of water and 99 µl of QIAseq Bead (Qiagen) containing magnetic beads for DNA purification were added to each sample. After 5 minutes of incubation at room temperature, the tubes containing the samples were placed on a magnetic stand and, after capturing the beads bound to the DNA, the supernatant was discarded. The beads were resuspended in 55 µl of water and after 5 minutes of incubation at room temperature, the tubes were placed on a magnetic stand. The supernatant, containing the DNA, was transferred to a new tube and a new purification cycle with beads was performed: 50 µl of magnetic beads were added to each sample, which were then incubated at room temperature for 5 minutes. Subsequently, the tubes were placed on a magnetic rack and the

supernatant was discarded. Beads were washed 2 times with 200 µl of 80% ethanol. Ethanol was removed and the beads were resuspended in 35 µl of water. After 5 minutes of incubation at room temperature, the tubes containing the samples were placed on a magnetic stand. The supernatant, containing the purified DNA, was transferred to a new tube. Then, the amplicons were submitted to a second PCR for insertion of barcodes (identifying sequences of each sample). For this purpose, a plate from the QIAseq 16S/ITS 96-Index I kit (HT Array) was used, in which each well of the plate has a different identifying sequence. For the reaction, 32.5 µl of each purified DNA sample was pipetted into a well of the HT Array plate, along with 12.5 µl of Master Mix from the QIAseq 16S/ITS Panel kit and 5 µl of water. The PCR was performed in an Eppendorf thermal cycler, model Mastercycler epgradientS, with the following conditions: 95°C for 2 minutes; 14 cycles of 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 2 minutes; final extension at 72°C for 7 minutes, and keeping the sample at 4°C until the moment of purification.

At the end of the amplification, 45 µl of QIAseq Bead (Qiagen) containing magnetic beads for DNA purification were added to each sample. After 5 minutes of incubation at room temperature, the tubes containing the samples were placed on a magnetic stand and, after capturing the beads bound to the DNA, the supernatant was discarded. Beads were washed 2 times with 200 µl of 80% ethanol. Ethanol was removed and the beads were resuspended in 30 µl of water. After 5 minutes of incubation at room temperature, the tubes containing the samples were placed on a magnetic stand. The supernatant, containing the purified DNA, was transferred to a new tube. Libraries were quantified by real-time PCR using the NEBNext Library Quant Kit for Illumina (New England Biolabs). Real-time PCR was performed on the Eppendorf® realplex4 Mastercycler epgradientS equipment. After quantification, libraries were diluted to 4 nM and pooled to generate a pool of samples. The sample pool was denatured and diluted to 10 pM. Next, it was sequenced on the MiSeq equipment (Illumina), using the MiSeq 600 cycles v3 sequencing kit (Illumina) and following the manufacturer's protocol.

2.4 - Statistical analysis and bioinformatics of 16S rRNA gene sequencing

The FASTQ files generated by the sequencing, as well as the metadata containing the patients' clinical information, were imported into the QIIME2 2022.2.0 analysis software (40) on the Linux Ubuntu operating system (18.04.6). The DADA2 algorithm was applied to remove noise and low-quality sequences, promote sequence dereplication and filter out chimeras. Reverse reads were cleaved at the 220 bp position due to the drop in quality from

that position. After quality filtering, the forward and reverse readings were joined by overlapping the 3' regions. Similar sequences were grouped to generate an ASV-type count table (Amplicon Sequence Variant). The taxonomic analysis was performed using the SILVA SSU 99% database, version 138. For each sample, the relative frequencies of each taxon present in different taxonomic categories were obtained. Frequency matrices were imported into R to generate barplots using the ggplot2 package. The QIIME2 software was also used to access the alpha diversity in different groups, calculated using the Shannon diversity index. The non-parametric Kluskal-Wallis statistical test was applied to verify differences between 3 or more variables. For comparison of 2 variables, the Wilcoxon non-parametric test was applied. In both cases, a mean difference was considered when $p < 0.05$. To generate boxplot-type graphs, the data were imported into R using the ggplot2 package. The Principal Components Analysis was performed using the QIIME2 software, from distance matrices generated by the unweighted Unifrac method (unweighted Unifrac), and the PERMANOVA statistical test was applied. For differential abundance analysis between the control and SS groups, the LefSE analysis (Segata et al, 2011) was used through the Galaxy platform (21). To consider a taxon differentially abundant, $p < 0.01$ and LDA score > 3.0 were used.

3 – Results

3.1 - Demographic characteristics, clinical and periodontal parameters

A total of 70 samples were initially collected (SS group = 35, control group = 35). However, after DNA quantification by real-time PCR, 09 samples were excluded due to insufficient genomic material to proceed with sequencing. Thus, a total of 61 samples were sequenced, 27 for the SS group and 34 for the control group. Demographic characteristics, clinical and periodontal parameters are described in **Table 1**. The mean age of the patients in the SS group was 50.52 years, while the mean age in the control group was 50.85. In both groups, the predominance was female.

Regarding the use of oral prosthesis, we observed that patients in the SS group had a higher percentage (58.30%) of oral prosthesis use, meaning they had a greater need for oral rehabilitation, compared to the control group (62%). Furthermore, mean DMFT, plaque index and presence of periodontal pockets greater than 3.5 mm were more prevalent in the SS group compared to the control group (Table 1).

Table 1. Demographic characteristics and comparison of clinical oral examinations between SS and Control groups

Group	**SS (n =27)**	**Control (n = 34)**	***P* value**
Age (mean)	50.52 y	50.85 y	0.63
Sex (%)	Female 25 (92.59%) Male 2 (7.41%)	Female 31 (91.18%) Male 3 (7.41%)	1.00
Oral Prosthesis (%)			
No	10 (41.70%)	17 (65.38%)	
FPP	7 (29.17%)	4 (15.39%)	0.193
RPD	2 (8.33%)	2 (7.69%)	
CD	5 (20.80%)	3 (11.54%)	
DMFT (mean)	22.32	17.75	
PI (%)			
0	2 (8.8%)	0	
1	1 (4.4%)	11 (57.9%)	<0.001
2	10 (43.4%)	4 (21.05%)	
3	10 (43.4%)	4 (21.05%)	
PSR (%)			
0	1 (4.35%)	6 (27.3%)	
1	3 (13.04%)	3 (13.6%)	
2	9 (39.13%)	9 (40.9%)	0.049
3	9 (39.13%)	2 (9.1%)	
4	1 (4.35%)	2 (9.1%)	

SS: group with Sjogren's Syndrome; y: years; FPD: fixed partial denture; RPD: removable partial denture; CD: complete denture: DMFT: missing, decayed and filled teeth; PI: plaque index; PSR: periodontal screening and recording index.

3.2 – Microbial diversity

In the analysis of microbial diversity by relative abundance, there was a significant difference between both groups (p 0.034). The classification by phyla showed similarities between both groups, with the phyla *Actinobacteriota*, *Bacteroidota*, and *Fusobacteriota* being more abundant in the control group, while the phylum *Firmicutes* was more abundant in the SS group (Figure 1). In the analysis by genus, there was a discrepant abundance between

different genera, being more abundant in the control group *Haemophilus*, *Neisseria* and *Prevotella*. While for the SS group, the genera *Stenotrophomonas*, *Streptococcus* and *Veillonella* were found in greater abundance (Figure 2).

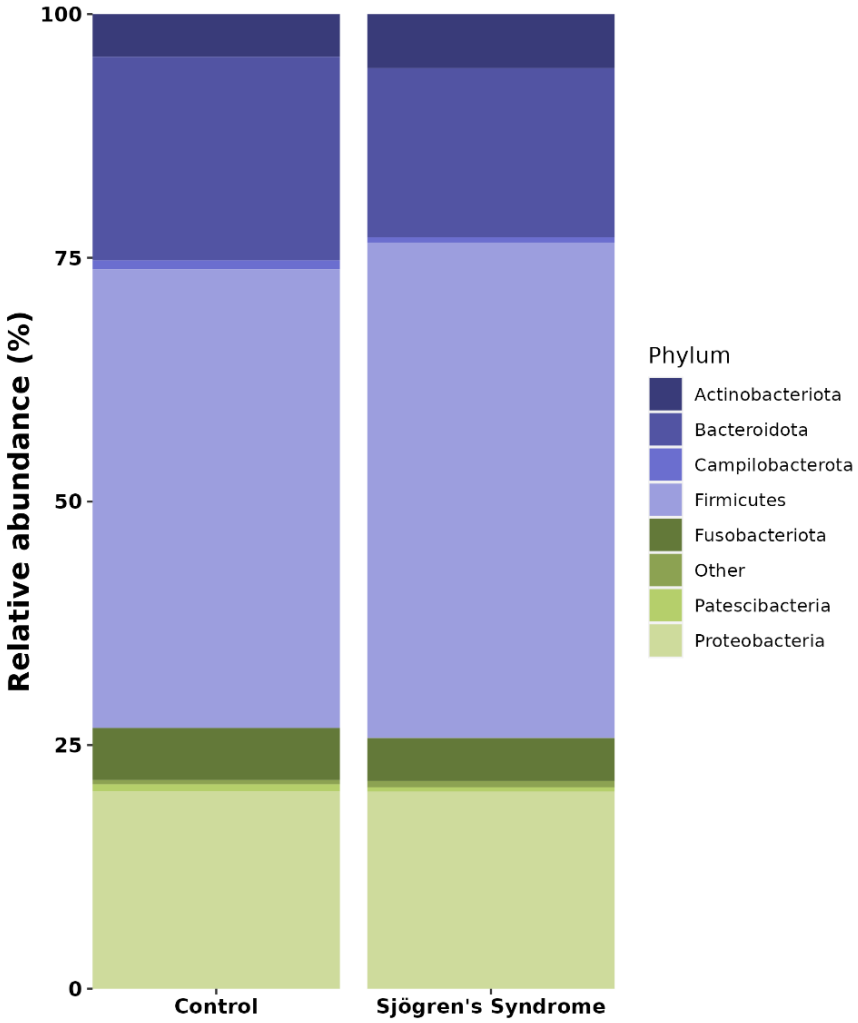

Figure 1. Relative abundance by phylum in the control and SS groups

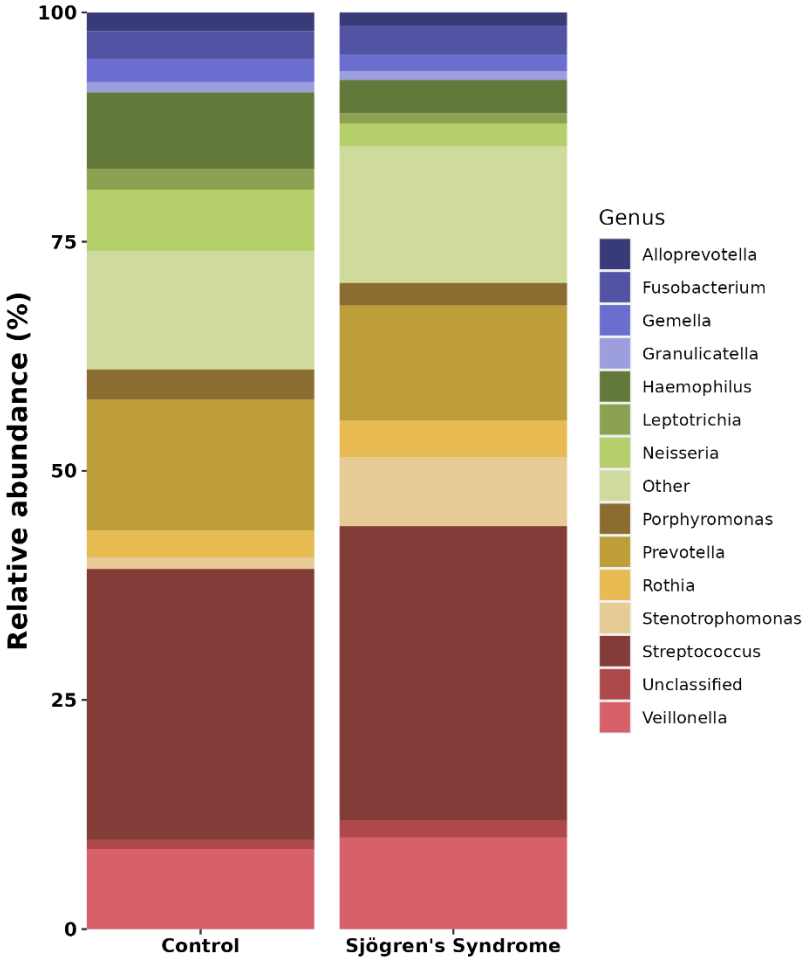

Figure 2. Relative abundance by gender in the control and SS groups

3.3 – Oral Microbiota and Periodontal Parameters

Regarding the analysis of bacterial diversity in relation to PSR, the SS group presented lower bacterial diversity when compared to the Control group, with the exception of the controls with PSR 3 and 4, which showed similar abundances to those in the SS group (Figure 3). For the Plaque Index, there was a significant difference between both groups (p 0.03), patients in the SS group with an index of 3 had the lowest bacterial diversity when compared to the bacterial diversity of indices 0, 1 and 2 in the SS and Control groups (Figure 4).

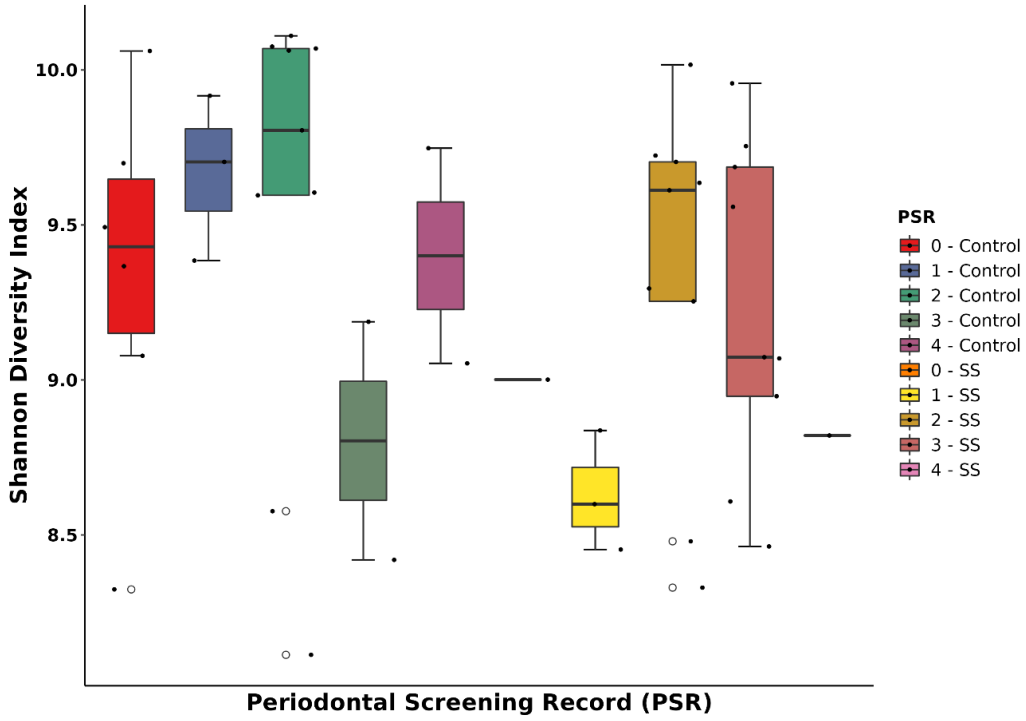

Figure 3. Bacterial alpha-diversity (Shannon index) of total saliva microbiota compared to PSR in control and SS groups

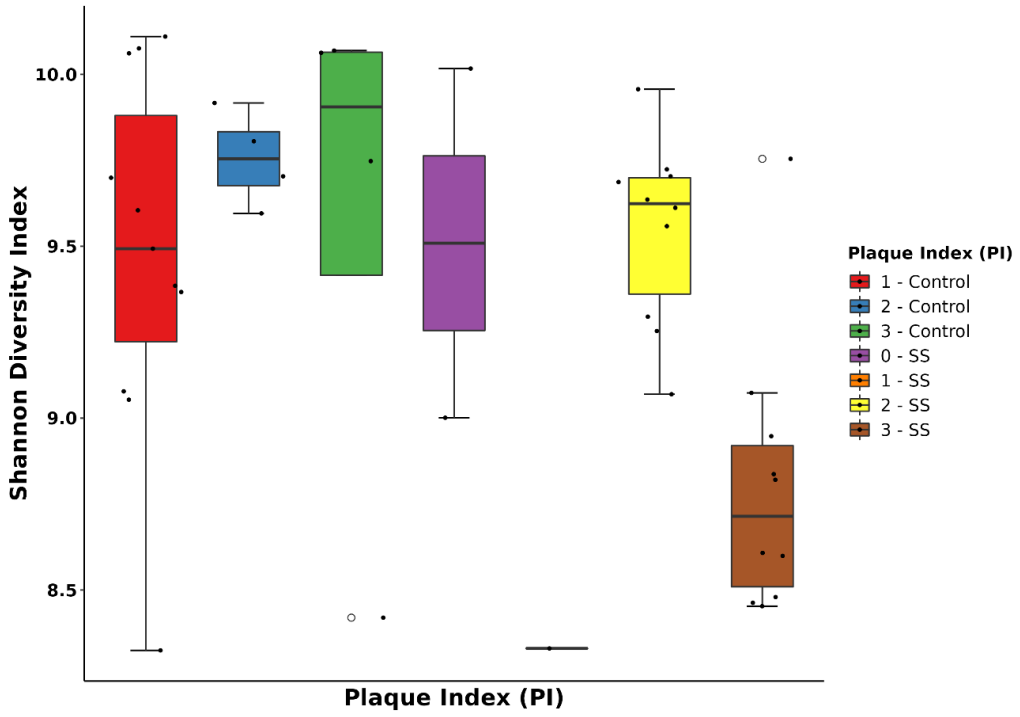

Figure 4. Bacterial alpha-diversity (Shannon index) of total saliva microbiota compared to PI in control and SS groups

3.4 – Oral microbiota and salivary flow

Regarding the NSSF, it was considered as hyposalivation when the patient had a volume lower than 1.5/15 minutes (19) (Table 3). The SS group presented a lower NSSF as expected, with 76.93% of the patients with a salivary flow between 0 and 0.25 mL/min. When comparing NSSF and bacterial diversity, patients in the SS group with lower NSSF had a greater bacterial diversity, compared to patients in the SS group who had a higher NSSF. However, the bacterial diversity of the SS group was lower when compared to the control group independently of the NSSF result (p = 0.25) (Figure 5).

Table 3. Comparison of Unstimulated Salivary Flow (NSSF) between groups with SS and Control

Group	SS	Control	*P* value
NSSF (ml/min)			
1	20 (76.93%)	5 (27.78%)	<0.001
2	6 (23.07%)	13 (52.22%)	

ml/min: milliliters per minute 1: 0 – 0.25 ml/min; 2: 0.26 – 1.00 ml/min;

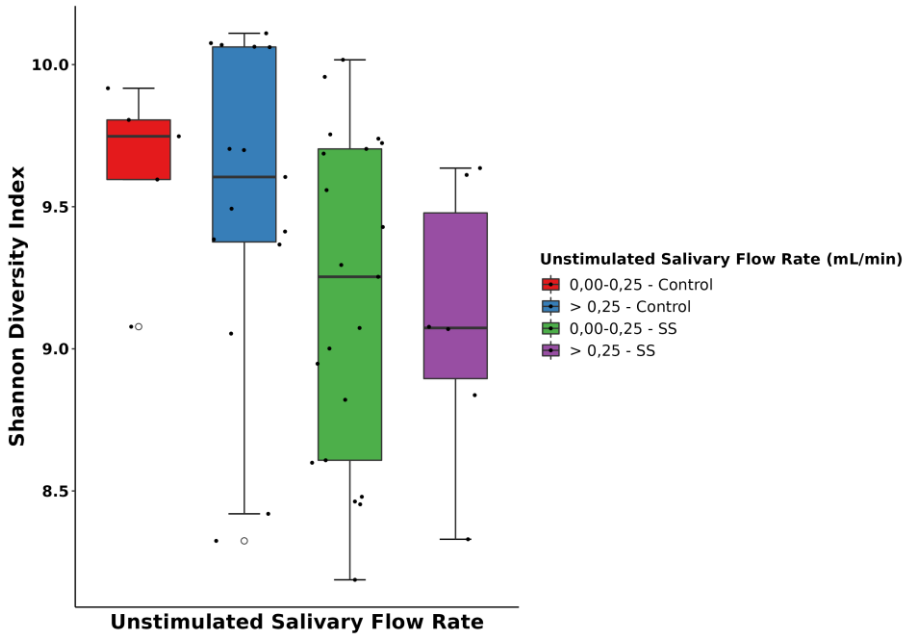

Figure 5. Bacterial alpha-diversity (Shannon index) of total saliva microbiota compared to NSSF in control and SS groups

3.5 – Time of evolution of SS and FS

With reference to the time of evolution of the disease for patients in the SS group, despite not showing a significant difference (p= 0.74), patients with a time of evolution of less than 40 months had a greater bacterial diversity, compared to those with a longer time disease progression (Figure 6). Regarding FS, most SS patients presented between FS > 1. When comparing bacterial diversity and FS, patients with FS = 2 and 3 showed the lowest bacterial diversity among the SS group (Figure 7).

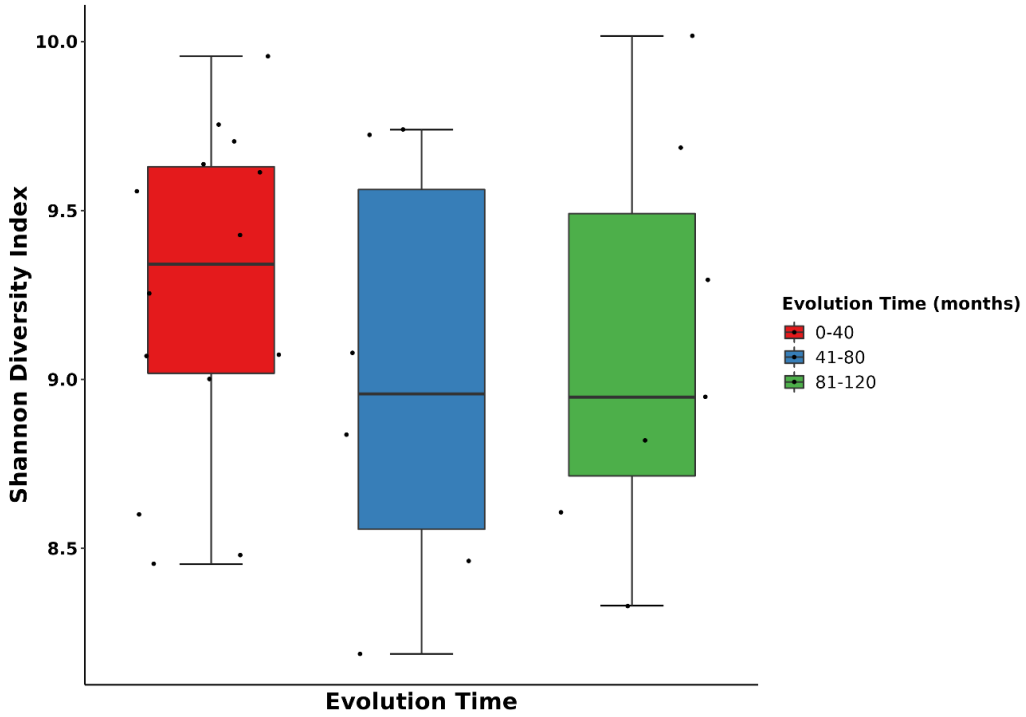

Figure 6. Bacterial alpha-diversity (Shannon index) of total saliva microbiota compared to time of evolution of SS.

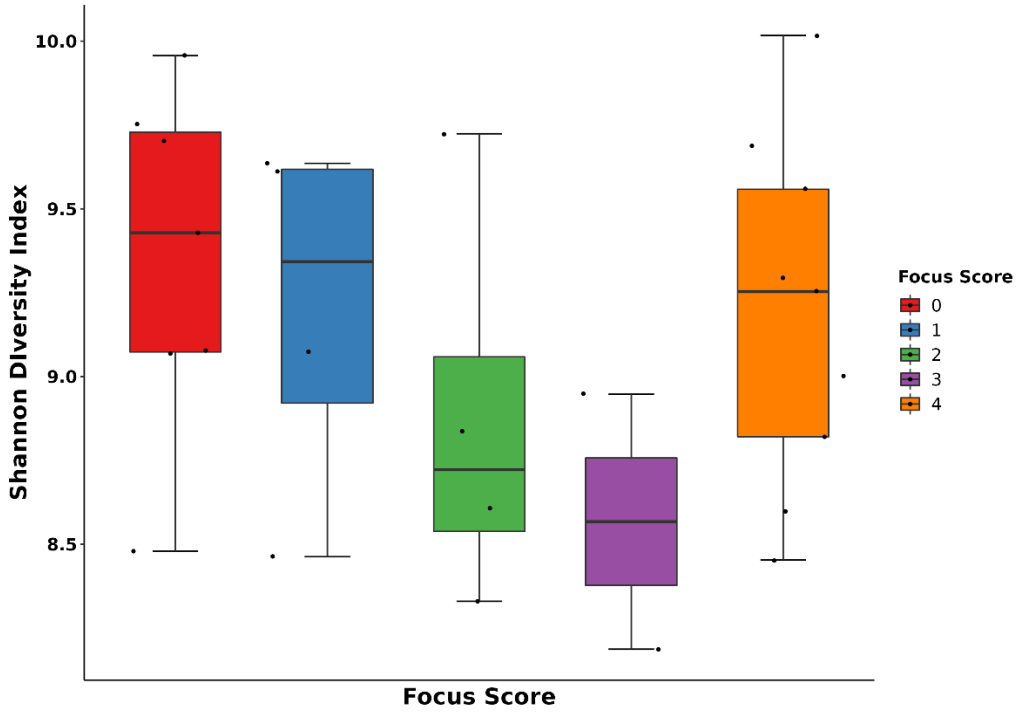

Figure 7. Bacterial alpha-diversity (Shannon index) of total saliva microbiota compared to Focus Score in SS group.

3.6 – Bacterial diversity of the Candida Colony Forming Units (CFUs) and chromogenic culture medium (CHROMagar) in control and SS and groups

The SS group presented more positives to CFUs and selective chromogenic culture medium (CHROMagar) in comparison to controls, with 29.63% (n=8) of the patients above 800 CFUs. The most common coloration of CHROMagar was green (*C. albicans*) for the SS group, and an association of green and pink for the control group (Table 4).

In the bacterial diversity index, the patients of the SS group that presented less than 800 CFUs showed significantly lower bacterial diversity than controls with the same CFUs count (p = 0.02). No statistical difference was found for the 800-uncontable CFUs between both groups (p = 0.63).

In regard to chromogenic culture medium (CHROMagar) and bacterial diversity, statistical difference was found for the patients with green coloration (*Candida albicans*), with the control group showing higher bacterial diversity in comparison to SS group (p= 0.02), and for the pink coloration (*Candida glabrata)*, with SS group showing higher bacterial diversity than controls (p= 0.04)

Table 4. Comparison of CHROMagar and Colony-Forming Units (CFU) of SS and Control groups

Group	SS	Control	*P* value
CHROMagar			
Negative	6 (22.22%)	11 (32.35%)	
C. tropicalis	1 (3.70%)	1 (2.94%)	
C. albicans	8 (29.65%)	14 (41.18%)	
C. glabrata	2 (7.40%)	7 (20.59%)	0.027
2 colors*	9 (33.33%)	1 (2.94%)	
3 colors**	1 (3.70)	0 (0%)	
CFUs			
1	19 – (70.37%)	31 – (91.18%)	0.048
2	8 – (29.63%)	3 – (8.82%)	

*2 colors: *C. albicans* and *C. glabrata*; *C. albicans* and *C. tropicalis*; **3 colors: *C. albicans*, *C. glabrata*. and *C. tropicalis*; 1: 0 – 800 CFUs; 2: 800 – Uncountable CFUs

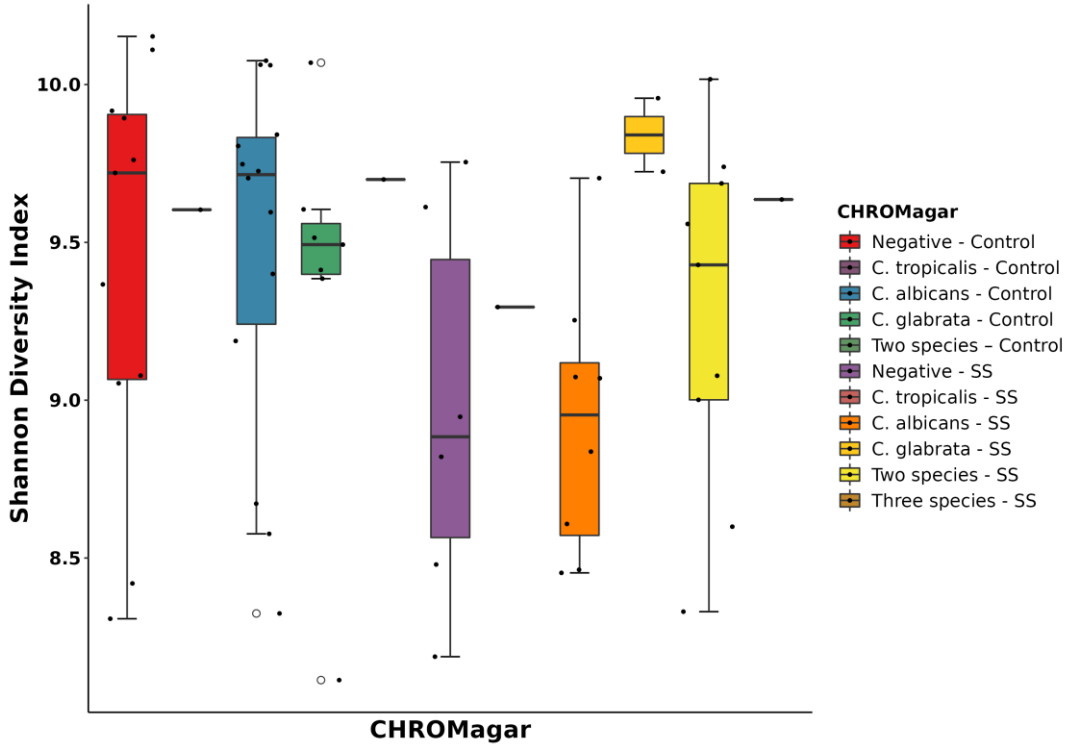

Figure 8. Bacterial alpha-diversity (Shannon index) of total saliva microbiota compared to CFU

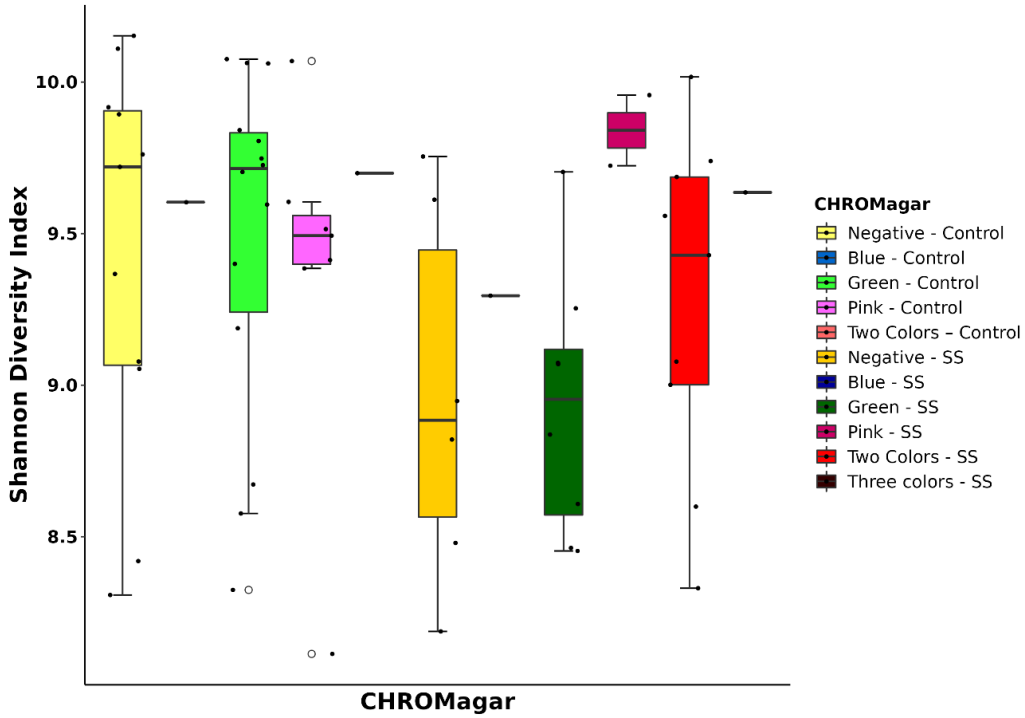

Figure 9. Bacterial alpha-diversity (Shannon index) of total saliva microbiota compared to CHROMagar

4 – Discussion

The present study assessed the oral microbiota of patient with SS, and the association of microbiological findings with the parameters PSR, NSSF, DFMT index, CFUs and chromogenic culture medium results. The latter were analyzed in comparison to bacterial diversity. Our samples were primary collected from female patients (n= 25, mean age 49.24 years) diagnosticated with SS. This result was expected since SS has been previously been reported as a disease more prevalent among females of mean age (1,22).

We found that SS was associated with reduced bacterial alpha diversity in whole saliva samples as compared with the control group. In this matter, most recent studies are in agreement with our results (7,25–29), presenting reduced bacterial diversity and dysbiosis in SS patients, nonetheless one reports was found showing no difference between SS patients from controls regarding oral microbiome (6). The reduced bacterial diversity in the SS patients may be related to the reduced salivary flow, which can alter the composition of the oral microbiota, and decrease the development of the microbiome of the soft and hard tissues of the mouth (30). This was also observed between our results, since 76.93% of the SS patients presented low NSSF (31). Regarding the bacterial diversity, SS groups presented reduced diversity in comparison to the control group, even in SS patients with normal salivary

flow. (24) The study of Siddiqui et al. (2016) presented similar results, demonstrating that oral dysbiosis in SS patients can occur in patients with normal salivary flow, being the microbial changes in SS not related to salivary flow (12).

The comparative analysis of clinical parameters between both groups showed that SS patients presented higher DMFT index (mean 22.32), simultaneously with a higher use of dental prosthesis (n= 14). High caries rate in SS patients has been previously reported (28,30), specifically in the root surfaces, which can be related to the higher loss of teeth in SS patients. The SS patients in our study also presented higher PI results when compared to controls, concurrently associated with a higher reduction in bacterial diversity. Higher PSR in both groups was associated with similar reduced bacterial diversity. Periodontal disease has been reported as significantly higher in SS patients and a positive association between both diseases has been hypothesized. (32) Also, high PSR and PI could be associated with low salivary flow in SS patients, due to decreased properties of saliva, such as its protective function (removal of bacteria and products of bacterial metabolism). (33)

In the correlation of characteristic bacteria per group, three genera were differentially abundant in the SS group: *Stenotrophomonas, Streptococcus* and *Veillonella*, while in control groups the genera *Haemophilus, Neisseria* and *Prevotella* were more prevalent. These genera, with the exception of *Stenotrophomonas* and *Neisseria*, are commonly found in the oral microbiota. Bacteria of the genus *Stenotrophomonas* are infections, commonly associated with an untreated infection, but they can also be related to periodontitis, which justifies their presence in the sample. The genus *Streptococcus* is regularly found in the oral cavity, but when associated with a diet rich in sugars, there is a significant increase in bacteria of this genus, starting to be registered with diseases of the oral tract, such as dental caries, periodontitis, and, more severe, may be associated with involved abscesses and infective endocarditis. Bacteria of the genus *Veillonela* are associated with the presence of periodontitis. Bacteria of the genus *Prevotella,* which are commonly found in subgingival plaque, may be associated with cases of periodontal disease, dentoalveolar abscesses and para-endodontic diseases. The genus *Haemophilus* is present in the oral cavity, both in the oral mucosa, as well as in saliva and on the tooth surface, being bacteria of low pathogenicity. The genus *Neisseria* is at a low level in the oral cavity, but can contribute to the anaerobic condition in the initial dental plaque formation, favoring the succession of anaerobic microorganisms, which may explain its prevalence in the control group. *Lactobacilli were* also founded in higher abundance in SS patients than in controls. This genus has been

founded as prevalent in the supragengival biofilm (22). It is a bacteria associated with low salivary flow, which justifies its high prevalence in patients with SS.

The study of Sembler-Møller et al., 2019 presented a different result. They found no statistical difference in the genera and species composition between SS patients and healthy controls (23). This may be associated with the state of oral health, since in their study, there was no significant difference between the rates of caries, with similar dental condition between the test and control groups, different from our results.

In regards to SS characteristics, including disease evolution time and FS, SS patients with over 40 months of disease diagnosis presented reduced bacterial diversity, when compared to SS patients with shorter time of diagnosis. Patients with focus score of 1 to 4 presented lower bacterial diversity in comparison to patients with FS of 0. FS of >1 has been strongly associated with abnormal NSSF. FS refers to the degree of lymphocytic infiltration resulting in pathological changes in the minor salivary glands (34). There are no previous studies associating FS with oral bacterial diversity, however reduced salivary flow associated with FS >1 and fibrosis of the minor salivary glands has been related to high DMFT index score, (35) which could be associated with changes in the oral microbiome of SS patients.

Regarding the occurrence of oral candidiasis, the SS group presented higher results of CFUs and CHROMagar when compared to control group, being *C. albicans* the main strain founded (41.18%) among SS positive cultures. Bacterial diversity was statistically lower in the SS groups when compared to number of CFUs. Controls with <800 CFUs presented higher bacterial diversity than SS with <800 CFUs. SS has been considered a high-risk disease for oral candidiasis due to decreased salivary flow, and re-establishment of the oral microbiome symbiosis has been reported as an alternative treatment to reduce *Candida* spp colonization in SS patients (36).

5 – Conclusion

Differences of oral microbiota between SS and control group were identified in this study. Bacteria of the genus *Stenotrophomonas*, *Streptococcus* and *Veillonella* were the most prevalent in the SS group, while bacteria of the genus *Haemophilus*, *Neisseria* and *Prevotella* were the most prevalent in the control group. A less diverse microbiota was found in SS patients, in association with higher scores in clinical index such as DMFT, PSR and PI. A reduced microbiota was also found in SS patients independently of the salivary flow score. These findings support the hypothesis of a swift in oral microbiome of SS patients that occurs independently of the alterations in the production of saliva by salivary glands.

6 – References

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APPENDIX

PARECER CONSUBSTANCIADO DO CEP

DADOS DA EMENDA

Título da Pesquisa: Análise da condição bucal e microbiota oral de pacientes com Síndrome de Sjögren

Pesquisador: Ana Carolina Fragoso Motta
Área Temática:
Versão: 5
CAAE: 95703618.4.0000.5419
Instituição Proponente: Universidade de Sao Paulo
Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 5.646.216

Apresentação do Projeto:
Síndrome de Sjögren (SS) é uma doença sistêmica autoimune, de evolução crônica, caracterizada por infiltração linfocítica das glândulas exócrinas, e secura de olhos e boca. Em relação à cavidade oral, a maioria das manifestações têm como causa primária a hipofunção das glândulas salivares e consequente hipossalivação. Além destes sintomas, cárie, doença periodontal e infecções fúngicas, como a candidíase, são consideradas as principais complicações orais secundárias. Apesar das evidências de que alterações na microbiota oral possam estar associadas à hipossalivação, pode-se considerar que os distúrbios autoimunes são preditores independentes em casos de candidíase. Por conseguinte, este trabalho pretende determinar a condição de saúde oral e a população microbiana oral de pacientes com SS primária e secundária. Pacientes em atendimento no Ambulatório de Doenças Oculares Externas, da Divisão de Oftalmologia do Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto - USP para tratamento da SS, durante o período de outubro de 2018 a setembro de 2019, e que concordarem e atenderem aos critérios de inclusão, participarão do estudo. Serão investigadas informações relativas à idade, sexo, etnia, forma clínica, secura de boca e olhos, exames laboratoriais específicos para o diagnóstico da SS e uso de medicamentos por meio da revisão dos prontuários médicos. Apenas os casos que foram diagnosticados de acordo com os critérios do Grupo de Consenso Americano-Europeu (2002) serão incluídos no estudo. Além destes dados clínicos relativos à SS, será realizado exame físico da cavidade bucal, onde será avaliado o fluxo salivar não-estimulado, índice de biofilme dentário,

a condição periodontal (Periodontal Screening and Recording - PSR) e de dentes (dentes cariados, perdidos e obturados - CPO-D), e a presença de candidíase. Amostras da microbiota oral obtidas por meio de enxaguado bucal serão congeladas e submetidas à técnica do pirosequenciamento do gene 16S, para determinação da população microbiana. O grupo controle consistirá de indivíduos referenciados à FORP-USP para tratamento odontológico de rotina, pareados por sexo e idade. Este estudo será submetido ao Comitê de Ética em Pesquisa da FORP-USP, e os pacientes deverão dar o consentimento livre e esclarecido. Os resultados deste trabalho possibilitarão o estabelecimento de condutas preventivas e terapêuticas a este grupo de pacientes.

Objetivo da Pesquisa:

Objetivo Primário:

Determinar a população microbiana oral, assim como a condição bucal de pacientes com SS primária e secundária.

Objetivo Secundário:

-Comparar a população microbiológica oral de pacientes com SS primária e secundária;-Correlacionar os achados microbiológicos com: xerostomia, hipossalivação, condição dental (CPOD), periodontal (PSR) e ocorrência de candidíase oral.

Avaliação dos Riscos e Benefícios:

Riscos:

Os riscos previsíveis à saúde em consequência desta pesquisa são mínimos, como por exemplo, discreto incomodo após cuspir a saliva e desconforto relacionados aos exames periodontal (sondagem); isso volta ao normal poucos minutos depois da coleta.

Benefícios:

Como benefícios, os exames que serão realizados ajudarão a determinar a condição bucal e microbiota oral de pacientes com a Síndrome de Sjögren, e assim métodos preventivos para evitar quaisquer problemas orais.

Comentários e Considerações sobre a Pesquisa:

O pesquisador responsável relata o atual estágio da pesquisa:

"Para o projeto de pesquisa serão coletadas amostras de enxaguado bucal com PBS (Solução tampão de Fosfato) de pacientes com e sem diagnostico de Síndrome de Sjögren. Será realizado exame físico da cavidade bucal, fluxo salivar não-estimulado, índice de placa, condição periodontal (Periodontal Screening and Recording - PSR) e de dentes (dentes cariados, perdidos e obturados - CPO-D), e presença de candidíase. Os pacientes com síndrome de Sjogren (SS) foram

recrutados dos pacientes em atendimento no Ambulatório de Doenças Oculares Externas, da Divisão de Oftalmologia do Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto – USP; e os pacientes do grupo controle foram recrutados dos pacientes referenciados à Faculdade de Odontologia de Ribeirão Preto – USP
(FORP-USP) para tratamento odontológico de rotina, os quais foram pareados por sexo e idade com os pacientes do grupo SS.
Foi realizada triagem e captação de pacientes com e sem diagnostico de Síndrome de Sjögren; coleta de enxaguado bucal com PBS de ambos os grupos; processamento das amostras para obtenção de precipitado para análise de DNA bacteriano; treinamento laboratorial para extração de DNA; extração do DNA das amostras de biofilme oral do grupo teste (com síndrome de Sjgren) e grupo controle (sem síndrome de Sjogren). Atualmente está sendo finalizada a extração de DNA das amostras coletadas do grupo controle, e posteriormente será realizada a amplificação por PCR, sequenciamento das amostras amplificadas de ambos os grupos, e analise bioestatisticas dos resultados obtidos."

O pesquisador responsável solicita a inclusão de duas pesquisadoras.
Solicita também a prorrogação de prazo devido a atrasos decorrentes da Pandemia de COVID-19

Considerações sobre os Termos de apresentação obrigatória:
O cronograma foi devidamente atualizado e as duas novas pesquisadoras inseridas na equipe do projeto na Plataforma Brasil

Recomendações:
Não há

Conclusões ou Pendências e Lista de Inadequações:
Emenda aprovada.

Considerações Finais a critério do CEP:
Emenda aprovada conforme deliberado na 262ª Reunião Ordinária do CEP/FORP de 15/09/2022.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_1953747_E2.pdf	08/08/2022 06:53:15		Aceito
TCLE / Termos de	TCLE_Controles_08_08_2028.pdf	08/08/2022	Ana Carolina	Aceito

Continuação do Parecer: 5.646.216

Assentimento / Justificativa de Ausência	TCLE_Controles_08_08_2028.pdf	06:51:21	Fragoso Motta	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_Sindrome_de_Sjogren_08_08_2028.pdf	08/08/2022 06:51:04	Ana Carolina Fragoso Motta	Aceito
Projeto Detalhado / Brochura Investigador	Projeto_detalhado_08_08_2028_com_novo_cronograma.pdf	08/08/2022 06:48:12	Ana Carolina Fragoso Motta	Aceito
Outros	Formulario_para_EMENDA_SS_08_08_2022.pdf	08/08/2022 06:47:14	Ana Carolina Fragoso Motta	Aceito
Outros	Formulario_Relatorio_Parcial_SS_08_08_22.pdf	08/08/2022 06:46:52	Ana Carolina Fragoso Motta	Aceito
Folha de Rosto	Folha_de_rosto_SS.pdf	12/08/2021 11:23:07	Ana Carolina Fragoso Motta	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

RIBEIRAO PRETO, 15 de Setembro de 2022

Assinado por:
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Folha de Informação

Em consonância com a Resolução CoCEx-CoG n° 7.497/2018, informamos que a Comissão de Graduação da Faculdade de Odontologia de Ribeirão Preto da Universidade de São Paulo (FORP/USP) em sua 531ª Reunião Ordinária, realizada em 03 de junho de 2024, **aprovou,** fundamentando-se na sugestão da Subcomissão para Avaliação dos Trabalhos de Conclusão de Curso (TCCs) da Unidade, **a inclusão deste trabalho na Biblioteca Digital de Trabalhos Acadêmicos da USP (BDTA).**

Cumpre-nos destacar que a disponibilização deste trabalho na BDTA foi autorizada pelos autores (estudante e docente orientador), conforme menção constante no trabalho e documentação existente no Serviço de Graduação da FORP.

Ribeirão Preto, 03 de junho de 2024.

Prof. Dr. Michel Reis Messora
Presidente da Comissão de Graduação
FORP/USP