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Original Article

# Oral microbiota in xerostomia patients - A preliminary study

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## KEYWORDS

Microbiota;  
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**Abstract** *Background/purpose:* The estimated prevalence of xerostomia (lack of saliva) ranges from 10% to 50% of the general population. The oral cavity provides a multivariant environmental habitat to over 700 species of bacteria and fungi. We hypothesized that xerostomia will alter the composition of oral microbiota.

*Material and methods:* Nineteen xerostomia patients and 10 healthy normal volunteers were studied for the oral microbiota. Gingival plaques were collected and microbiota were detected using bacterial 16S ribosomal RNA and analyzed based on the levels of phylum and class.

*Results:* In all cases, phyla of Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, and Proteobacteria make up to 100% of oral microbiota at phylum level. Analyzing individual phylum, presence of Bacteroidetes in xerostomia patients and normal subjects were  $23.12 \pm 2.56\%$  and  $23.23 \pm 2.58\%$ , respectively. Mean percentage presence of Firmicutes phylum in xerostomia patients and normal subjects were  $18.94 \pm 1.83\%$  and  $14.06 \pm 0.98\%$ , respectively. Statistically significant difference was not observed between xerostomia patients and normal subjects in this study.

*Conclusion:* These observations revealed obvious but not statistically significant changes in oral major microorganism phylum between xerostomia patients and normal subjects in this

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study. More samples are needed to verify the current results and to use oral microbiota as a tool in the diagnosis of xerostomia.

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## Introduction

Xerostomia is common in many medical conditions such as Sjogren's syndrome. The estimated prevalence of this symptom ranges from 10% to 50% of the general population.<sup>1</sup> Human saliva is composed of over 98% water with the remainder consisting of electrolytes, mucin, antibacterial substances, and enzymes. This later but minor parts control the growth of oral microorganisms and maintain an oral microflora balance.<sup>2</sup> The oral cavity provides a multivariant environment habitat to over 700 species of bacteria and fungi.<sup>3</sup>

Oral microbiota is one of the most complicated but easily accessed microorganisms in humans. Along the surfaces in oral cavity, such as teeth, gingiva, tongue, pharynx, and buccal mucosa, all can form microorganism colonies because continuous salivation provides nutrients for the growth of microorganisms.<sup>4</sup> Alongside with caries and periodontitis, many systemic diseases have been proposed to relate to oral microbes, including oral squamous cell carcinomas, rheumatoid arthritis, diabetes mellitus, Crohn's disease and obesity.<sup>5–7</sup>

The microbial population, different from normal constitution, are apparent in several pathologic conditions.<sup>7</sup> Thus, we hypothesized that a change in the amount of saliva will alter the composition of oral microbiota.

## Materials and methods

### Sampling

Enrolled in this study were 19 patients with xerostomia, including those diagnosed with Sjogren's syndrome (N = 12) and non-Sjogren's xerostomia (N = 7) by the inclusion criteria and exclusion criteria as below:

#### Inclusion criteria

- A. Dry mouth with a diagnosis of Sjogren's syndrome.
- B. Dry mouth without a diagnosis of Sjogren's syndrome but with a history of taking medications showing dry mouth side effects.
- C. Otherwise healthy, without any other medications and habits, such as antibiotics or smoking.

#### Exclusion criteria:

- A. Patients taking antibiotics or smoking.
- B. Unwilling to participate in the study.
- C. Taking hormonal therapy.

None of these patients were in any medical treatment for dry mouth other than daily home care. Ten healthy volunteers were also recruited for the study. Ages are 23–58 y/o in control group with median of 35.5 (N = 10) and 25–83 y/o in xerostomia group with median of 58 (N = 19). Xerostomia group was composed of Sjogren's and non-Sjogren's subgroups. Sjogren's subgroup patients were all diagnosed carrying catastrophic illness card. The recruitment followed the approved guideline by the Institutional Review Board at Chung Shan Medical University Hospital. Gingival plaques were collected as comprehensively described in the core microbiome sampling protocol A of Human Microbiome Project Manual (Core Microbiome Sampling Protocol A, HMP Protocol # 07–001, Version:12.0, 29 Jul 2010, 7–3). In brief, gingival plaques were collected, placed in PowerBead Tube (Qiagen, Germantown, MD, USA) and stored at 4 °C until further analysis. Microbiota were detected using bacterial 16S ribosomal RNA and were analyzed based on the levels of Phylum and Class.

### Sample DNA extraction and measuring DNA concentration

All samples were immediately placed in ice after collection, transferred to –20 °C storage by the end of the collection day, and maintained at –20 °C for maximum of a month at the field site before transfer to long-term storage at –80 °C in the laboratory. DNA was extracted using the DNeasy PowerSoil Kit manufactured (Qiagen). The isolation procedure was performed according to the manufacturer's standard protocol. The DNeasy PowerSoil Kit is used in the standard operating protocol of both the Human Microbiome Project and the Earth Microbiome Project, and samples stored in a preservation buffer provided in the kit as PowerBead Tubes, contain 750 µl solution. After recovery, DNA was measured using a NanoDrop 2000C spectrophotometer (Thermo Scientific, Waltham, MA, USA) and stored at –80 °C.

### Library construction and sequencing for V4 region of 16S ribosomal DNA

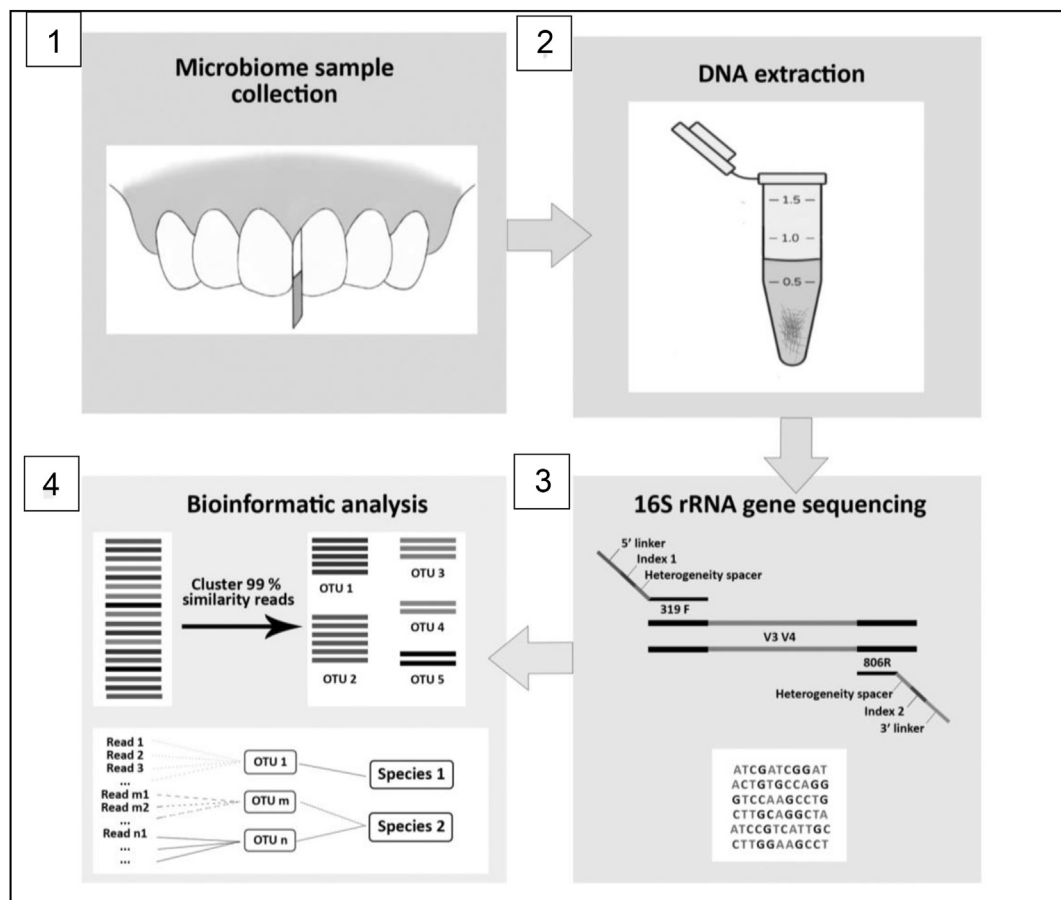
The PCR primers, F515 (5'-GTGCCAGCMGCCGCGGTAA-3') and R806 (5'-GGACTACHVGGGTWTCTAAT-3'), were designed to amplify the V4 domain of bacterial 16S ribosomal DNA. The primer set was added Illumina Nextera adapter sequence on the 5'-end for library preparation step. PCR amplification was performed in a 50 µl reaction volume containing 25 µl 2X Phusion HF Master Mix (New England Biolabs, Hertfordshire, UK), 0.5 µM of each forward and reverse primer, and 50–150 ng DNA template.

The reaction conditions consisted of an initial 98 °C for 30 s, followed by 30 cycles of 98 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s, as well as a final extension of 72 °C for 5 min. Next, amplified products were checked by 2% agarose gel electrophoresis and ethidium bromide staining. Amplicons were purified using the AMPure XP Beads (Agencourt Bioscience, Beverly, MA, USA), and quantified using Nanophotometer (Implen GmbH, Munchen, Germany) - all according to respective manufacturers' instructions. For library preparation, Illumina Nextera Index Primer kit was used to create library. Purified libraries were QC and quantified again by 2% agarose gel electrophoresis, Qubit (Thermo Scientific) and qPCR method. Finally, libraries were normalized to same concentration and sequencing by Miseq sequencer (Illumina, Inc., San Diego, CA, USA).

### Bioinformatics analysis

We used the QIIME2-2019.10 platform to perform the microbial analysis.<sup>8</sup> Raw 16S rDNA gene sequences were demultiplexed using the q2-demux pipeline. The sequences were then denoised and filtered PhiX reads and chimeric sequences with DADA2 (via q2-dada2).<sup>9</sup> It was

then merged to single-end sequences using the DADA2 plugin, in which, sample metadata containing the information such as mice type, treatment, and various clinical parameters for categorical and numerical formatting was used. For trimming and truncating using the DADA2 plugin to remove low-quality regions of sequences, the filter parameters were set up at 19 and 214 for left forward read (R1) and 20 and 156 for right forward read (R2). To create a feature table generation, two plugins were then conducted using feature-table summarize and feature-table tabulate-seqs in QIIME2. To construct a phylogeny, all amplicon sequence variants were aligned with mafft (via q2-alignment).<sup>10</sup> Alpha-diversity metrics including observed features and Faith's Phylogenetic Diversity were calculated using q2-diversity. Sequences were clustered using VSEARCH plugin (q2-vsearch) into operational taxonomic units (OTUs) for each sample with a 99% sequence similarity cutoff value.<sup>11</sup> A summary of all taxonomic information was generated using the q2-feature-classifier classify-sklearn naïve Bayes taxonomy classifier against the Silva data set version 138.<sup>12,13</sup> To standardize results, the equivalent number of sequence reads (based on the lowest number of sequences obtained from a single sample) per

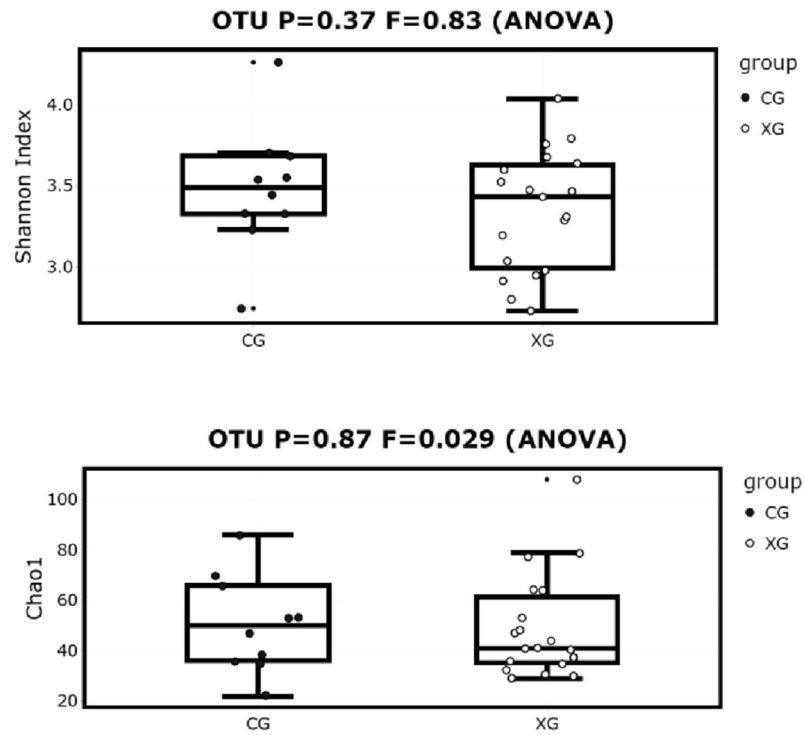


**Figure 1** Methodology flow chart. 1. Gingival sample collection after acquiring patient informed consent signature in clinical trial. 2. DNA extraction follows the guidance of DNeasy PowerSoil Kit manufactured by Qiagen. 3. 16S rRNA sequencing by Illumina Miseq platform. 4. Bioinformatic analysis: cluster all sequencing reads within 99% similarity to pick OTUs (operational taxonomic units), and then the OTUs are compared against database to form taxonomy assignment.

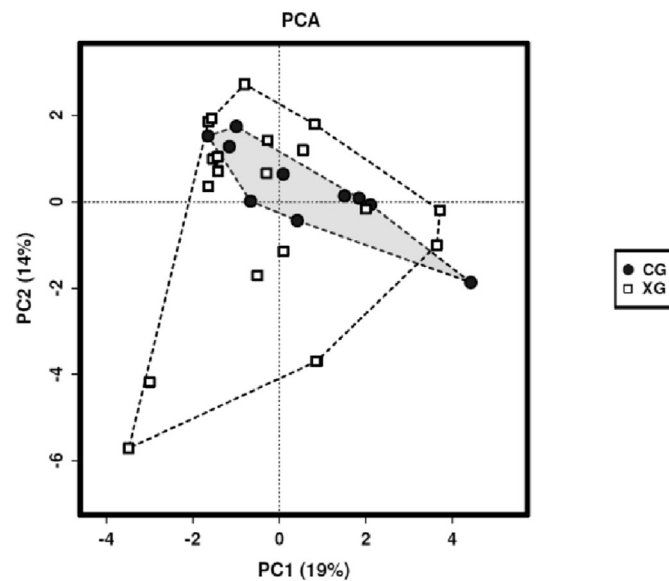
**Table 1** Operational taxonomic units (OTUs) classification and classification status identification.

OTUs	Kingdom	Phylum	Class	Order	Family	Genus	Species
OTU without database available	0	2	3	8	46	269	269
OTU within database	453	451	450	445	407	184	184

(A)



(B)



**Figure 2**  $\alpha$ - and  $\beta$ -diversity analysis between xerostomia group and control group. (A) Shannon and Chao1 Index of xerostomia group (XG, N = 19) and control group (CG, N = 10). Black square point is the mean of each group. (B) PCoA analysis between xerostomia group (XG) and control group. Circle point represents XG, square point represents CG.

sample that was chosen by rarefaction were performed for all subsequent comparisons. To determine the core microbiome, genus abundance  $>0.1\%$  was used for analysis. Venn diagrams were constructed using Venny 2.1. Both matrices for the complete and the re-sampled datasets were calculated and compared by applying the Mantel tests implemented in the R (version 3.6.3) package Vegan. For beta-diversity analysis, we determined the microbial composition diversity between the individuals using weighted UniFrac, unweighted UniFrac, Jaccard distance, and Bray–Curtis dissimilarity in q2-diversity plugin.<sup>14,15</sup> The linear Principal Component Analysis (PCA) model was also performed using q2-diversity. For featured taxa selection, we used LEfSe and Calypso to calculate the linear discriminant analysis effect size (LEfSe) and random forest prediction,<sup>16</sup> in which, LDA score of  $>3.0$  and the Kruskal–Wallis test ( $\alpha$ -value of 0.05) were set as thresholds. To perform microbial function analysis, we used the phylogenetic investigation of community by reconstruction of unobserved states (PICRUSt) to investigate.<sup>17</sup>

The study flow chart is comprehensively described in Fig. 1.

## Results

The bacterial distribution by classification level: xerostomia group vs healthy group.

Based on qualified sequencing data, a total of 453 bacterial operational taxonomic units (OTUs) were obtained from all samples, 184 OTUs with validly named species. The distribution by classification level is as Table 1. Three hundred and eighty-four OTUs were obtained from xerostomia group (valid  $N = 19$ ) and 240 OTUs in control group (valid  $N = 10$ ) suggesting more abundant OTUs in xerostomia group. Among 384 OTUs of xerostomia group, 156 OTUs have validly named species, while 122 OTUs of control group without validly named species.

Xerostomia group has a greater number of species than control group, though the total richness showed in Shannon Index and Chao1 Index did not show significant difference between the two groups (Fig. 2A). Although the microbiota composition seemed to be more complex in xerostomia group by PCoA analysis, there was no statistically significant difference (Fig. 2B).

In the microbiota composition analysis, there was a shift in phylum level despite the diseases, Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, and Proteobacteria being the most abundant microorganisms in oral microbiota.

Mean percentage presence of Actinobacteria in xerostomia and normal subjects were  $22.33 \pm 3.14\%$  and  $23.26 \pm 3.98\%$ , respectively, while those of Bacteroidetes in xerostomia and normal subjects were  $23.12 \pm 2.56\%$  and  $23.23 \pm 2.58\%$ , respectively. Mean percentage presence of Firmicutes phylum in xerostomia and normal subjects were  $18.94 \pm 1.83\%$  and  $14.06 \pm 0.98\%$ , respectively. In the Fusobacteria phylum, the mean presence in xerostomia and normal subjects were  $11.55 \pm 1.48\%$  and  $13.95 \pm 1.79\%$ , respectively. Mean percentage presence of Proteobacteria phylum in xerostomia and normal subjects were  $16.64 \pm 1.49\%$  and  $16.12 \pm 1.33\%$ , respectively. There was

**Table 2** Comparison of microbiota composition between xerostomia and control groups at the phylum level.

Taxonomy	Xerostomia (N = 19) (Mean $\pm$ SD)	Healthy volunteer (N = 10) (Mean $\pm$ SD)
p_Actinobacteria	$22.33 \pm 3.14\%$	$23.26 \pm 3.98\%$
p_Bacteroidetes	$23.12 \pm 2.56\%$	$23.23 \pm 2.58\%$
p_Firmicutes	$18.94 \pm 1.83\%$	$14.06 \pm 0.98\%$
p_Fusobacteria	$11.55 \pm 1.48\%$	$13.95 \pm 1.79\%$
p_Proteobacteria	$16.64 \pm 1.49\%$	$16.12 \pm 1.33\%$

no statistical significance between xerostomia group and healthy volunteer group, which is shown in Table 2.

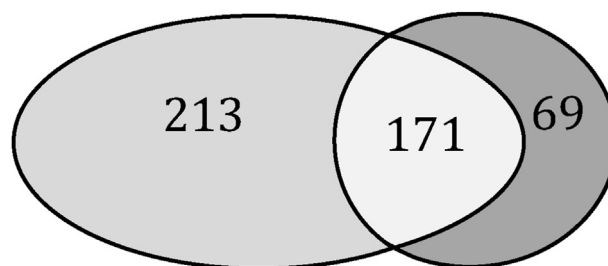
As regard to the ratio of Firmicutes and Bacteroidetes, the study results showed 0.82 and 0.61 in xerostomia and healthy volunteer groups, respectively. Although the major composition changes have been shown at Actinobacteria phylum, Firmicutes phylum and Proteobacteria phylum, there has been no statistical significance (t-test).

We further analyzed the core microbiota between xerostomia and healthy volunteer group. Xerostomia group shared the common 171 OTUs (Fig. 3) with healthy volunteer group. Among those, 66 OTUs were with validly named species. In terms of sequence reads analysis, the core share of OTU reads between xerostomia and healthy volunteer group occupied nearly 90%.

## Sjogren's and non-Sjogren's syndrome comparison

When we compared samples of Sjogren's syndrome and non-Sjogren's syndrome in xerostomia group, the percentage presence of every phylum is shown in Table 3. No statistical significance was noted among the groups.

The major different phyla between Sjogren and non-Sjogren groups were Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria. In core microbiota analysis, the common OTUs occupied total sequence reads of Sjogren's and non-Sjogren's sub-groups were 85% and 90%, respectively. However, the common OTU number was only about 28% of total observed OTUs.



**Figure 3** The common (171) and unique operational taxonomic units (OTUs) between xerostomia group (XG, total number of OTUs in XG is  $171 + 213 = 384$ ) and control group (CG, total number of OTUs in CG is  $171 + 69 = 240$ ). The light gray part (213) is the unique number of OTUs of xerostomia. The dark gray part (69) is the unique number of OTUs of control group. The white part is the core share OTUs.

**Table 3** Comparison of gingival microbiota composition between Sjogren's and Non-Sjogren's subgroups at phylum level.

Taxonomy	Sjogren's (N = 12) (Mean ± SD)	Non-Sjogren's (N = 7) (Mean ± SD)
p_Actinobacteria	24.53 ± 4.50%	14.93 ± 0.86%
p_Bacteroidota	24.03 ± 3.46%	26.62 ± 8.70%
p_Firmicutes	16.78 ± 2.14%	22.89 ± 10.25%
p_Fusobacteriota	11.69 ± 1.80%	13.81 ± 4.88%
p_Patescibacteria	3.28 ± 0.78%	4.10 ± 2.70%
p_Proteobacteria	15.45 ± 1.93%	10.79 ± 3.94%

## Discussion

Human saliva is produced by three major salivary glands, including parotid, submandibular and sublingual glands as well as thousands of minor salivary glands. These glands produce about 1–1.5 L of saliva every day. Saliva produced by parotid glands was 70% serous, and 30% mucous in nature, while half and half in submandibular gland, and mostly mucous in sublingual glands. The components of saliva were mainly water (about 98%), and the rest included electrolytes, mucoproteins, enzymes lysozymes, lactoperoxidases, lactoferrin and immunoglobulin A, which all together can control the growth of microorganisms and maintain a stable oral microbiota.<sup>18,19</sup>

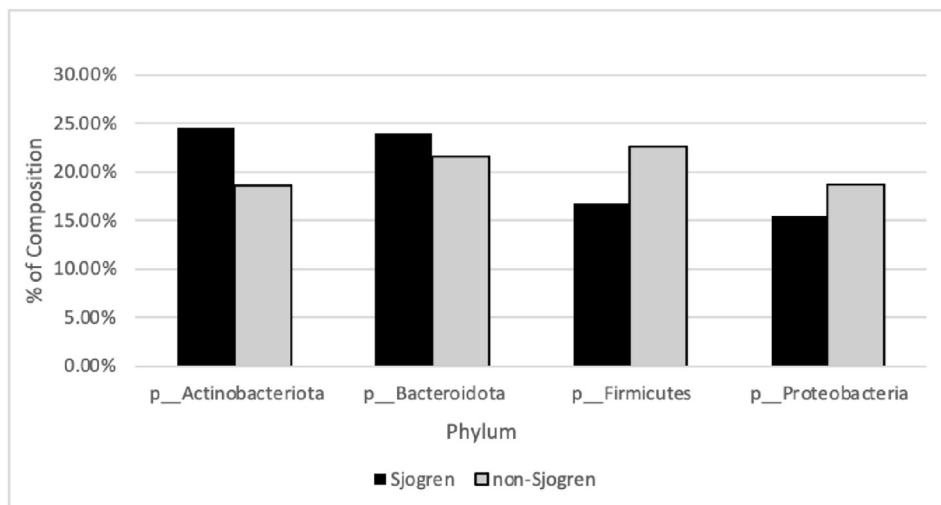
Xerostomia is a commonly seen clinical symptom with an overall estimated prevalence of 22% in general while there is a higher prevalence in elderly people. Up to 30% of females and 50% of older people have been shown to have dry mouth all over the world.<sup>1,20</sup> The autoimmune diseases act as an etiology for dry mouth, though medications including anticholinergic, sympathomimetic, or diuretics, cause most of the xerostomia. Sjogren's syndrome is an autoimmune disease with symptoms including dry mouth and dry eyes.<sup>21</sup> It is known to be a B-cell lymphocyte

related disease with a predilection of females (9:1) and prevalence of 0.1%–4.8%.<sup>22</sup> In Taiwan, the incidence of Sjogren's syndrome is 6 cases per 100 thousand population with a female to male ratio of 9.9:1.<sup>23</sup> Since the saliva volume might influence oral microbiota, we hypothesized that the presence of an autoimmune Sjogren's syndrome condition may alter the oral microbiota. However, as shown in Fig. 4, no statistical significance was found between the different groups, but we noticed major composition changes at p\_Actinobacteria, p\_Firmicutes and p\_Proteobacteria which may be of clinical interest and diagnostic value in the future (Table 2).

Among the diverse microbial community in the human gastrointestinal tract, Firmicutes is the largest phylum in gut microbiome and is suggested to be involved in the development of diabetes and obesity.<sup>24–26</sup> While Bacteroides are commonly discovered in the human intestine and a significant portion of the fecal bacterial population, no major shifting in oral microbiota composition was found in our study.

Human gut microbiota is composed predominantly of two phyla, Bacteroidetes and Firmicutes.<sup>27</sup> Studies showed that implanting adult germ-free mice with normal gut microbiota produces an increase of 60% body fat in these lean animals and that the implanted animals carry more Firmicutes than the lean ones.<sup>28</sup> These investigational results suggested a major role of Firmicutes in fatty acid absorption and lipid metabolism.<sup>29</sup>

Populations in Bacteroidetes phylum are most anaerobic and are resistant to many antibiotics. Most of them are docile inhabitants when they remain in the GI tract but become virulent when they escape from the GI tract. In humans, Bacteroides species' main source of energy is fermentation of a wide range of sugar derivatives from plant material. These compounds are common in the human colon and are potentially toxic. Bacteroides converts these sugars to fermentation products, which are beneficial to humans. Bacteroides also have the ability to remove side chains from bile acids, thus returning bile acids to the hepatic circulation.

**Figure 4** Major different microbiota phylum in Sjogren's and non-Sjogren's syndrome.



In conclusion, the main phylum difference between xerostomia and healthy groups was Actinobacteria, Firmicutes, Fusobacteria and Proteobacteria. The same phylum shift was also noted between Sjogren's and non-Sjogren's syndrome. These observations may provide a useful diagnostic tool for clinical practice. However, a major limitation in this study was the sample size analyzed. In future studies, more samples will be used to verify the findings of this preliminary study.

## Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

## Acknowledgement

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