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Original Article

Oral specimens as a tool for accurate metagenomic analysis: A pilot study

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ABSTRACT

Objectives: Acute oral mucosal damage, as well as other inflammatory processes seem to be related to dysbiosis of the oral microbiome. The need to study changes in the oral microbiome led us to hypothesize what type of sample would provide the most representative picture of the entire human oral microbiome.
Materials and methods: An observational, and cross-sectional study was carried out. Six healthy adult participants provided 3 different sample types each, that included saliva, oral rinse and mucosal biopsy tissue. We performed 16S rRNA sequencing of the V3-V4 region of the 18 samples using Illumina MiSeq technology.
Results: Participants were 27 ± 6,3 years old. Bacterial alpha diversity was higher in oral rinse samples compared to whole unstimulated saliva and oral mucosa tissue (p = 0,005). However, saliva specimens showed a 56 % relative abundance of identified species followed by a 30 % in oral rinse and only 1 % in tissue samples.
Conclusions: This study found differences on oral microbiome composition for each type of sample. Oral rinse should be chosen when higher alpha diversity is needed, whereas whole unstimulated saliva should be more appropriate for larger amount of bacterial DNA.
Clinical relevance: The results obtained demonstrate the importance of a correct choice of the optimal type of oral sample for microbiome studies due to the differences found in its composition.

1. Introduction

Acute oral mucosal damage is a frequently reported side effect in patients treated with systemic antineoplastic agents [1-2]. Its overall prevalence is 38,2 % [3], and its development is believed to have a multifactorial origin [4], where oral microbiota of the host seems to be a relevant component [5]. The study of changes in the oral microbiome composition may help to reveal certain biological mechanisms that may be amenable to intervention in order to prevent or treat this condition. This imbalance of the community structure of the microorganisms within the oral cavity is known as dysbiosis and appears to be closely related to local inflammatory processes [6]. The term oral microbiota refers to all microbes inhabiting the oral cavity including bacteria, fungi, archaea and viruses, meanwhile the oral microbiome also comprises their genes [2,5,7]. In most cases, the word microbiome is used imprecisely when meaning bacteriome, since it is the most studied and known of the biomes [5]. Although the oral microbiome is considered a single complex entity, evidence shows that its composition can substantially differ among oral anatomical locations within the same individual [8-9]. Multiple techniques have been used throughout the years to identify microorganisms; however Next Generation Sequencing has been accepted as the most reliable technology [5,10-12]. Within this method, shotgun metagenomics has proved to be useful when profiling taxonomic composition [13–14]. Despite the technological advances achieved so far, there are still difficulties in the taxonomic identification of the different species of microorganisms. Previous studies have analysed numerous types of samples from the oral cavity that include supra- and subgingival dental plaque; tongue, palate and retropharyngeal swabs, whole unstimulated saliva as well as oral rinse and mucosa [9,15-16]. Logical thinking leads us to believe that many of these samples, such as swabs and dental plaque, due to their method of collection, provide us with information on subpopulations of microorganisms. Nevertheless, acute oral mucosal damage onset might occur at any region of the oral cavity [1]. Hence, we designed a preliminary verification study to determine the optimal sample type to allow the most accurate metagenomic analysis.

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2. Material and methods

2.1. Study design and study population

This study was conceived as an observational, descriptive, and cross-sectional pilot study. Every participant was recruited at the Clinic of the Faculty of Dentistry by MPS. All the subjects were offered to participate, and those that met eligibility criteria were voluntarily enrolled. Inclusion criteria was as follows: healthy subjects who have not been diagnosed with or are not suffering from systemic diseases as well as not receiving treatments that alter the immune system [13]. All of them were ≥ 18 years old and able to understand and agreed to participate. On the other side, subjects could not enrol if they were receiving anticoagulants or antiaggregant, had previous allergic reaction to local anaesthetic drugs, were unable to comprehend the implications of the study, not willing to participate or unable to give written consent. Furthermore, subjects were not eligible if they performed oral washout or brushed their teeth 12 h prior to collecting the samples. Moreover, candidates with decrease overall saliva rate were also excluded.

Participants' data were collected and registered in a coded database to avoid their identification through personal information and to ensure privacy. Also, three different samples were taken from each subject following a strict order to minimize possible interferences in the specimens: First whole unstimulated saliva, second oral rinse, and lastly an oral mucosa biopsy. In order to reduce risks and unnecessary invasive techniques such as the extraction of oral mucosa biopsy in healthy people, we chose to select candidates who required extraction of a third molar for non-infectious causes.

The present research was approved by the Galician Ethics Committee of Clinical Research (Ref. No. 2020/552) and conducted in accordance with the guidelines for Good Clinical Practice and the Declaration of Helsinki. All participants signed the informed consent form.

2.2. Specimen's collection

Each subject agreed to provide three different biological specimens for the study. Unstimulated whole saliva was the first sample obtained from patients by leaning their heads forward and letting saliva flow towards a sterile empty Eppendorf sample container until reaching a volume of 5 mL. Next, participants were asked to rinse mouth with 5 mL of 0,9 % sodium chloride (NaCl) solution for 1 min [16], ensuring they reached every part of the oral cavity with it and finally spitting it into a sterile empty sample container without any other medium. Finally, a biopsy of the oral mucosa was aseptically extracted by an odontologist who infiltrated local anaesthesia in the chosen area of the participant's oral cavity, a 5 × 5 mm tissue sample was removed with a scalpel and the resulting incision was sutured with silk to stop bleeding and prevent future infection. The selected fragment corresponded to tissue adjacent to the gingiva of the third molar. These tissue samples were collected into a sterile sample container with 0,9 % sodium chloride to avoid drying out of the specimen. All samples were stored frozen at −80 °C from collection, as subjects were enrolled, until all samples were collected for simultaneous processing.

2.3. DNA extraction, quantification and purity assessment

The isolation of DNA from all samples was carried out by using Maxwell® RSC PureFood GMO and Authentication Kit (Cat.# AS1600, Promega Corporation, Madison, WI) adhering to the manufacturer's instructions. Liquid samples such as whole saliva and oral rinse were centrifuged prior to DNA extraction treatment to increase the concentration of genetic material. Once supernatant was discarded, samples were re-suspended in lysis buffer (1 ml of CTAB), subsequently samples were processed following the same protocol as tissue samples. For solid samples, 150 mg of tissue were placed into a conical tube and 1 ml of CTAB Buffer was added followed by homogenization with a disposable pestle for 40 s. Resulting liquid was then transferred into a Lysing Matrix E bead beating tube and vortexed for 30 s. Next, samples were taken to 95 °C for 10 min and cooled down during 1 min at room temperature and vortexed once again. Then, 40μl of Proteinase K and 20μl of RNase A were added and mixed using a vortex followed by incubation at 70 °C for 10 min. Consecutively, RSC cartridges were prepared and placed in the rack according to manufacturer. Elution tubes with samples were filled with 100μl of Elution Buffers and placed in cartridge to, finally, run Maxwell® RSC with the PureFood GMO and Authentication Protocol. Extracted DNA samples were stored at −20 °C until further processing.

DNA concentrations were measured using the QuantiFluor® ONE dsDNA System (Promega Corporation, Madison, WI, USA) on a Quantus® fluorometer (Promega Corporation). In addition, purity was also evaluated as previously described [17]. DNA samples were frozen at −20 °C until further processing.

2.4. Library construction and 16S rRNA gene sequencing

Library generation was carried out through amplification of the V3 - V4 region of the 16S ribosomal RNA gene. Sequencing was performed on an Illumina MiSeq (Illumina, San Diego, CA) using the MiSeq v3 reagent kit. All samples were randomized and normalized to 5 ng/μL with PCR-grade water. Amplicons of approximately 460 bp were generated using primers from Integrated DNA Technologies, Inc. flanked by Illumina overhang adapters (Forward overhang: 5′TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG; Reverse overhang: 5′GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG.). The first PCR was carried out using a 25 μL reaction consisting of 5 ng/μL template DNA from 2X KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Wilmington, MA, USA). The thermal cycler was set as follows: 95 °C (3:00) + [95 °C(0:30) + 55 °C(0:30) + 72 °C(0:30)] x 25 cycles + 72 °C (5:00) and holding at 4 °C. For the second round of PCR, we added Illumina sequencing adapters as well as dual-index barcodes were added in a 50 μL PCR reaction from 5 μL of amplicon PCR product for 8 cycles using 2X KAPA HiFi HotStart ReadyMix using the same parameters [17].

Database used was Silva version 138_99. Illumina sequencing raw data are available in the BioStudies database (https://www.ebi.ac.uk/biostudies/) under accession number S-BSST1150.

2.5. Microbiome and statistical analysis

The sequencing data generated from the Illumina MiSeq sequencer was analyzed adhering to the methodology described in the literature [17–24] using the following software: BaseSpace Sequence Hub version: 7.11.0; Python version: 3.8.13; QIIME 2 version: 2022.2.0 [19]; q2cli version: 2022.2.0; FastQC v0.11.9 [20]; fastp 0.23.2 [21]; cutadapt 3.7 [22]; multiqc, version 1.12 [24].

2.6. Bioinformatic quality control measures

FastQC was carried out with default flags. Cutadapt was used to remove forward TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG and reverse GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC primers from sequences to analyze, following suggestions from Illumina 16S Metagenomics Sequencing Workflow (https://support.illumina.com/content/dam/illumina-marketing/documents/products/other/16s-metagenomics-faq-1270−2014−003.pdf), and rest of default flags.

Fastp was carried out with cutadapt output files and following flags (the rest are by default): trim_poly_x (enable polyX trimming in 3′ ends); cut_front −cut_tail (cutting extremes 5′ and 3′ of sequence,

drop the bases in the window if its mean quality < threshold, stop otherwise); cut_mean_quality=15 (the mean quality requirement option shared by cut_front and cut_tail); length_required=220 (reads shorter will be discarded); qualified_quality_phred=15 (means phred quality >=Q15 is qualified); unqualified_percent_limit=40 (40 % of bases are allowed to be unqualified). Alpha-rarefaction, from Qiime 2 tool, was used with flags by default.

3. Results

3.1. Characteristics of the study cohort

This pilot study included 6 participants (4 female and 2 male) with a mean age of 27 ± 6,3 years. Other sociodemographic data of the subjects are shown in Table 1. All of them were healthy subjects with caries, however none of them suffered from periodontitis, had implants, prosthetic teeth or were diagnosed with any medical condition of the gastrointestinal tract. Only 2 (33,33 %) of them were current smokers (of 5 cigarettes daily) at that time. In addition, 4 (66,67 %) out of the 6 participants had received systemic antibiotics a mean of 578,75 (± 475,27) days prior to enrolment, therefore this is not expected to alter the results of the oral microbiome analysis. Antibiotic treatments received by the participants are also displayed on Table 1.

3.2. Sequence reads and composition of the salivary microbiome

Each participant provided 3 samples, which means that the study comprised 18 specimens (Supplementary Table 1). The quality control analysis performed demonstrates that nearly all reads of the samples were in the range between 30 (0.1 % reading errors) and 40 (0.01 % reading errors) on the Phred Score Scale (Fig. 1a and Fig. 1b).

3.3. Quantitative metagenomic data

The mean of total reads in saliva samples was 265,452 ± 507,833,57 while in oral rinse was 209,772 ± 382,954,80 and in tissue specimens 57,936,17± 27,466,13 as detailed in Table 2. Saliva was the type of sample where a higher mean number of species were identified (638,00 ± 559,99), followed by oral rinse (618,17 ± 426,2) and lastly by tissue samples (404,67 ± 125,42).

According to phylum level, *Firmicutes* were the most abundant microorganism throughout all the specimens (55,57% in saliva, 55,25 % in oral rinse and 34,35 % in oral mucosa). *Bacteroidetes* were the second most abundant phylum (18,25 %) in saliva samples followed by *Actinobacteria* (13,4 %). However, *Actinobacteria* (15,54 %) and *Bacteroidetes* (13,08 %) rank second and third in terms of oral rinse abundance. Whereas 12,68 % of oral mucosa bacteria were unclassified at phylum level and 14,71 % were *Fusobacteria* (14,71 %) as shown in Supplementary Figure 1.

Focusing on the lowest taxonomic category, illustrated in Supplementary Figure 2, we found that in saliva samples the highest percentage were unclassified species (35,86 %) followed by *Rothia mucilaginosa* (5,81 %), *Granulicatella adiacens* (5,09 %), *Prevotella melaninogenica* (4,43 %) and *Actinomyces odontolyticus* (3,42 %). In oral rinse 39,23 % were unclassified, 5,36 % *Rothia mucilaginosa*, 3,65 % *Actinomyces odontolyticus*, 2,96 % *Gemella sanguinis* and 2,73 % *Streptococcus salivarius*. On the other hand, 43,76 % of species in tissue samples were unclassified, 2,65 % *Rothia mucilaginosa*, 0,79 % *Granulicatella adiacens*, 0,54 % *Porphyromonas pasteri* and 0,36 % *Prevotella melaninogenica*.

The data for the other classification levels are detailed in Table 2. In addition, graphical representations have been made, considering the five major components in each type of sample as well as their respective percentages in the other samples, at the class (Supplementary Figure 3), order (Supplementary Figure 4), family (Supplementary Figure 5) and genus levels (Supplementary Figure 6).

3.4. OTUs analyses of all samples were adequate

First, bacterial species count revealed that half of the samples (9 out of 18) contained between 0 and 300 Operational Taxonomic Units (OTUs). Three samples contained more than 1200 OTUs, 2 samples had between 600 and 900 OTUs and only 1 sample had 900–1200 OTUs as shown in Supplementary Figure 7. Data analysis led us to exclude 3 samples (S2, S3 and OR1) from the study because OTUs features could not be identified in them (Supplementary Figure 8).

In order to evaluate sequencing depth, a rarefaction plot [23,25] was built. Fig. 2 reflects that, although not all the types of specimens

Table 1
Socio-demographic and clinical cohort data.

Subjects		S1	S2	S3	S4	S5	S6	TOTAL	
								Mean (±SD)	%
Gender		F	M	F	F	M	F		66,67 (F), 33,33 (M)
Age		29	35	30	18	21	29	27(± 6,29)	
Marital status (single)		X	X	X	X	X	X		100
Occupation	Student	X			X	X			50
	Unemployed		X						16,67
	Worker			X			X		33,33
Education	Primary		X	X	X		X		66,67
	College	X				X			33,33
Smoker		X			X				33,33
Numb. Cig/day		5			5			5 (± 0)	
Caries		X	X	X	X	X	X	X	100
Periodontitis									0
implants									0
Prosthetic dental pieces									0
Active drug treatment		X		X					33,33
Type of drug treatment		Allergy shots		Anxiolytics; oral contraceptive					
Gastrointestinal (GI) Tract Medical Conditions									0
Systemic antibiotic use		X		X		X	X		66,67
Time from antibiotics (days)		383		252		1285	395	578,75 (± 475,27)	
Type of antibiotic		Amoxicillin trihydrate, potassium clavulanate		Amoxicillin		Levofloxacin	Amoxicillin		

Fig. 1. Quality control analysis of the sequencing reads. a. FastQC: Mean Quality Score. The analysis shows that almost all sequencing reads were between 30 (0,1 % reading error) and 40 (0,01 % reading error) on the Phred Score Scale according to the number of base pairs. b. FastQC: Per Sequence Quality Scores. The analysis shows that almost all sequencing reads were between 30 (0,1 % reading error) and 40 (0,01 %) on the Phred Score Scale according to the sequencing read counts.

have the same number of reads, each one of them reached a *plateau*, that indicates an adequate sequencing depth. Thus, it was observed that from 30 reads on, the quality of the samples was comparable.

Fig. 3 shows the relative frequency of OTUs distribution on each sample. Regarding tissue samples, DNA from 4 out of 6 samples could not be assigned to any specific bacterial species and the remaining two contained less than 10 % of DNA from known bacterial species. Only 1 out of 4 saliva samples had 100 % relative frequency of known bacterial species, 2 samples contained 50–60 % of known bacterial species' DNA and 1 sample contained purely unassigned bacterial DNA. Three out of 5 oral rinse samples were optimal (100 % known OTUs), one contained >35 % and only 1 of the samples had 100 % unassigned bacterial DNA.

3.5. *Alpha diversity was higher in oral rinse samples*

The complete phylogenetic analysis of all processed samples resulted in the circular cladogram displayed in Fig. 4a Subsequently, a Krona chart [26] was constructed for each type of specimen, aiming to exhibit the differences of the microbiome composition among them (Fig. 4b shows saliva analysis, Fig. 4c illustrates composition of oral rinse samples, and Fig. 4d represents tissue sample data). The information obtained manifests that about 44 % of bacteria in the saliva samples belongs to unassigned species, whereas in oral rinse samples this percentage is 70 %, reaching 99 % in tissue biopsy specimens.

We next examined alpha diversity by using Simpson's Index [27] which showed a p-value=0,005. Thus, a statistically significant difference was found between the alpha diversity of oral rinse versus unstimulated saliva and tissue biopsy (Fig. 5). Herein, bacterial alpha diversity is higher in oral rinse samples.

4. Discussion

Despite the large number of studies published on the oral microbiome to date, there is still no clear evidence to determine the optimal type of oral sample for an adequate analysis of alterations in its composition, and this is where the novelty of this study lies.

Considering the results obtained, we ought to expect low percentages of known bacterial species (<1 %) in oral mucosal tissue samples. Beforehand, this seems consistent with current knowledge, since it is known that most of the germs of the oral flora are simply

Table 2
Quantitative sequencing data from germs that constituted the five most abundant for each type of sample. Raw data obtained prior to processing for statistical quality purposes.

Type of sample	Saliva (Mean ± SD)	Oral rinse (Mean ± SD)	Tissue (Mean ± SD)
Number of Species Identified	638,00 ± 559,99	618,17 ± 426,2	404,67 ± 125,42
Total reads	265,452 ± 507,833,57	209,772 ± 382,954,80	57,936,17± 27,466,13
% of total Kingdom reads	99,95 ± 0,02	99,93 ± 0,13	87,64 ± 10,51
% of total Phylum reads	99,69 ± 0,1	99,65 ± 0,08	87,32 ± 10,4
% of total Class reads	98,41 ± 2,46	99,43 ± 0,12	87,10 ± 10,31
% of total Order reads	99,33 ± 0,15	99,33 ± 0,14	87,03 ± 10,33
% of total Family reads	98,99 ± 0,24	98,97 ± 0,19	86,34 ± 10,99
% of total Genus reads	98,24 ± 0,49	98,14 ± 0,56	85,22 ± 11,28
% of total Species reads	64,14 ± 4,24	60,77 ± 8,79	56,25 ± 20,27
% of Taxonomic_bacteria	99,95± 0,02	99,91 ± 0,05	87,61 ± 10,49
% of Taxonomic unclassified	0,05 ± 0,02	0,09 ± 0,05	12,36 ± 10,51
% of Taxonomic *Archaea*	0,00	0,00	0,14
% of Phylum *Firmicutes*	55,57 ± 5,27	55,25 ± 12,33	34,35 ± 20,11
% of Phylum *Bacteroidetes*	18,25 ± 5,52	13,08 ± 6,13	10,97 ± 7,19
% of Phylum *Fusobacteria*	5,30 ± 2,63	5,91 ± 6,03	14,71 ± 8,55
% of Phylum *Proteobacteria*	4,79 ± 2,83	7,65 ± 5,22	8,84 ± 7,4
% of Phylum *Actinobacteria*	13,40 ± 5,48	15,51 ± 7,09	11,14 ± 4,68
% of Phylum *Candidatus Saccharibacteria*	1,40 ± 0,99	1,30 ± 0,75	3,75 ± 4,54
% of Phylum *Absconditabacteria (SR1)*	0,32	0,19 ± 0,21	
% of Phylum *Unclassified*	0,31 ± 0,1	0,36 ± 0,08	12,68 ± 10,4
% of Phylum *Spirochaetes*	0,67 ± 0,73	0,78 ± 0,58	2,92 ± 3,56
% of Class unclassified	1,82 ± 1,35	2,09 ± 0,79	19,88 ± 10,81
% of Class *Bacilli*	43,95 ± 4,93	44,01 ± 11,95	26,83 ± 24,13
% of Class *Bacteroidia*	16,61 ± 5,48	11,19 ± 5,16	8,89 ± 6,13
% of Class *Fusobacteria*	5,30 ± 2,63	6,83 ± 6,25	14,71 ± 8,55
% of Class *Betaproteobacteria*	2,24 ± 1,05	4,46 ± 2,46	3,82 ± 3,88
% of Class *Clostridia*	4,49 ± 1,85	4,16 ± 2,14	7,42 ± 5,01
% of Class *Actinobacteria*	13,42 ± 5,5	15,51 ± 7,09	11,14 ± 4,68
% of Class *Negativicutes*	6,20 ± 4,63	6,83 ± 4,97	5,77 ± 3,38
% of Class *Gammaproteobacteria*	4,08 ± 1,48	3,58 ± 2,4	14,57
% of Order *Lactobacillales*	39,11 ± 3,78	37,91 ± 11,69	21,27 ± 24,36
% of Order *Bacteroidales*	16,61 ± 5,48	11,19 ± 5,16	8,21 ± 6,33
% of Order *Actinomycetales*	12,36 ± 5,75	14,22 ± 6,87	8,24 ± 5,79
% of Order *Selnomonadales*	6,20 ± 4,63	5,69 ± 4,97	4,81 ± 3,38
% of Order *Fusobacteriales*	5,30 ± 2,63	5,70 ± 6,25	14,71 ± 8,55
% of Order *Bacillales*	2,11 ± 0,32	2,19 ± 2,45	
% of Order *Clostridiales*	3,89 ± 1,97	3,15 ± 2,41	6,15 ± 4,99
% of Family *Streptococcaceae*	33,19 ± 6,34	33,86 ± 11,37	19,08 ± 20,89
% of Family *Prevotellaceae*	11,43 ± 5,05	7,96 ± 3,82	2,13 ± 0,57
% of Family *Micrococcaceae*	6,15 ± 5,67	7,47 ± 5,83	14,17
% of Family Unclassified			14,85 ± 10,37
% of Family *Carnobacteriaceae*	5,58 ± 2,66	2,58 ± 1,1	
% of Family *Veillonellaceae*	5,4 ± 4,6	5,69 ± 4,97	4,81 ± 3,38
% of Family *Bacillales Incertae Sedis XI*	3,74 ± 2,3	5,31 ± 3,82	
% of Family *Fusobacteriaceae*	1,77 ± 0,3	2,81 ± 6,22	9,38 ± 5,91
% of Family *Porphyromonadaceae*	4,93 ± 2,06	2,34 ± 0,67	5,01 ± 3,04
% of Genus *Streptococcus*	33,16 ± 6,40	33,81 ± 11,36	15,19 ± 15,50
% of Genus *Prevotella*	10,67 ± 4,40	7,61 ± 3,71	2,04 ± 0,67
% of Genus *Granulicatella*	5,54 ± 2,65	2,55 ± 1,08	
% of Genus *Fusobacteria*	1,76 ± 0,3	2,80 ± 6,2	9,36 ± 5,9
% of Genus *Veillonella*	4,86 ± 3,77	4,40 ± 4,25	
% of Genus *Actinomyces*	4,39 ± 1,05	5,19 ± 1,96	2,14 ± 1,27
% of Genus *Rothia*	3,87 ± 5,57	6,04 ± 4,72	2,34
% of Genus *Gemella*	4,06 ± 2,57	6,01 ± 3,54	
% of Genus Unclassified			14,78 ± 11,28
% of Genus *Leptotrichia*			4,3 ± 4,11
% of Species Unclassified	35,86 ± 4,24	39,23 ± 8,79	43,76 ± 20,27
% of Species *Granulicatella adiacens*	5,09 ± 2,58	2,28 ± 0,92	0,79 ± 1,26
% of *Prevotella melaninogenica*	4,43 ± 2,05	2,22 ± 1,41	0,36
% of Species *Porphyromonas pasteri*	2,99 ± 0,57	1.07 ± 1,42	0,54
% of Species *Actinomyces odontolyticus*	3,42 ± 0,48	3,65 ± 1,88	
% of Species *Gemella sanguinis*	2,35 ± 2,15	2,96 ± 3,33	0,12
% of Species *Gemella haemolysans*	2,15 ± 0,83	1,61 ± 0,57	
% of Species *Streptococcus salivarius*	1,89 ± 5,61	2,73 ± 6,1	
% Species *Rothia mucilaginosa*	5,81 ± 5,43	5,36 ± 4,51	2,65 ± 8,11

found in the oral biofilm lining the mucous membranes, therefore the rest of the sample tissue should presumably be bacteria free thanks to the protective function of the oral epithelium [28]. Thus, most DNA extracted from the sample should belong to the host and not to commensal microorganisms [29].

On the other hand, liquid samples like saliva and oral rinse have turned out to be quite different from each other. Saliva showed a 56 % relative abundance of identified species versus a 30 % found in oral rinse. In addition to this finding, it has been spotted that oral rinse statistically differed from saliva and tissue in terms of alpha

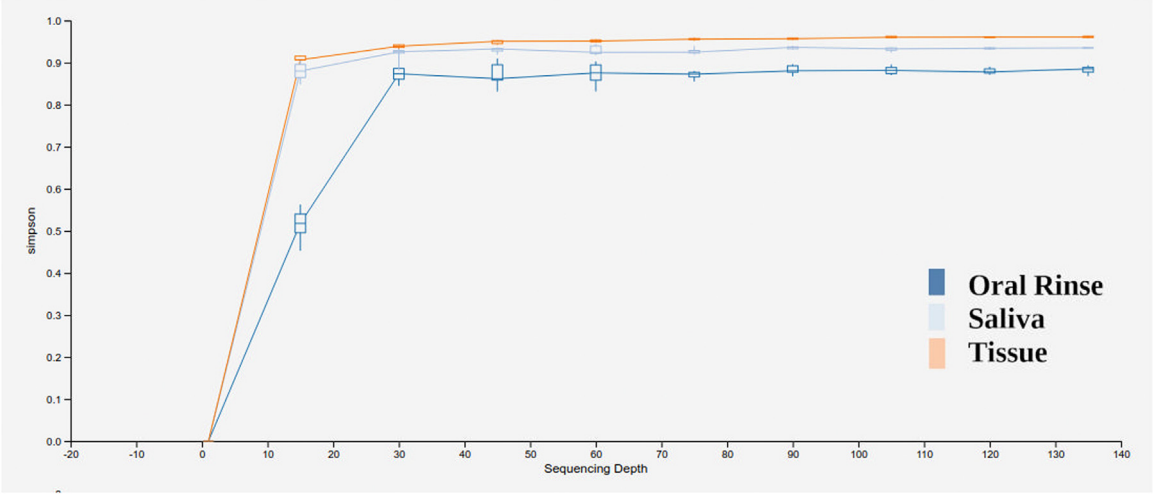

Fig. 2. Rarefaction curve plot. Simpson's Index analysis shows that from 30 reads on, the quality of the samples was comparable.

diversity, and therefore a higher microbial diversity was acknowledged. One of the hypotheses generated about this topic consists of the possibility that, the sample acquisition technique used for oral rinse may drag bacteria inhabiting different anatomical locations of the oral cavity. This phenomenon may not happen when obtaining unstimulated saliva as it simply flows, without stimulation, from the mouth into the sample collection container by gravity. In addition, the higher α diversity found in oral rinse within our study, compared to other specimens, also match previous investigations where oral rinse diversity was superior to heterogeneity detected with swabs collected from lesion and non-lesion sites [30]. Additionally, previous research has concluded that sample handling and processing can significantly affect the relative abundance of the identified microorganisms [31].

With regard to oral microbiome composition and given that our results showed superiority of saliva and oral rinse samples against mucosal tissue specimens, we compared our raw data with previously published studies. The main bacterial phyla identified in saliva samples were *Firmicutes* (55,57 %) similar to previous studies where authors identified 50–60 % [31]. The amount of *Bacteroidetes* differed in their relative abundance from our study (18,25 %) compared to <10 % in previous studies [32]. *Actinobacteria* relative abundance was slightly superior in our results (13,4 %) compared to a relative abundance close to 10 % in the literature [32]. *Fusobacteria* relative abundance was also comparable to previous results (5,30 %). Another study from the field identified the top five abundant genera in saliva samples to be *Prevotella, Neisseria, Streptococcus, Haemophilus* and *Rothia* [15] while in our results *Streptococcus, Prevotella, Granulicatella, Veillonela* and *Actinomyces* lead the ranking. In the case of oral rinse samples researchers found *Streptococcus, Prevotella, Neisseria, Haemophilus* and *Rothia* to constitute the majority of the genera [15], whereas we determined *Streptococcus, Prevotella, Rothia, Gemella* and *Actinomyces* to be the most prevalent genera in oral rinse samples.

Depending on the type of study we are planning to carry out, one sample is likely to be more appropriate than the other. In cases where we care about having a larger number of identified OTUs we would opt for a saliva sample, whereas if we are interested in analyzing more diverse populations of the oral biofilm, oral rinse would be the best choice. Knowing the best practice for oral bacteriome analysis and deciding the optimal specimen is fundamental when designing

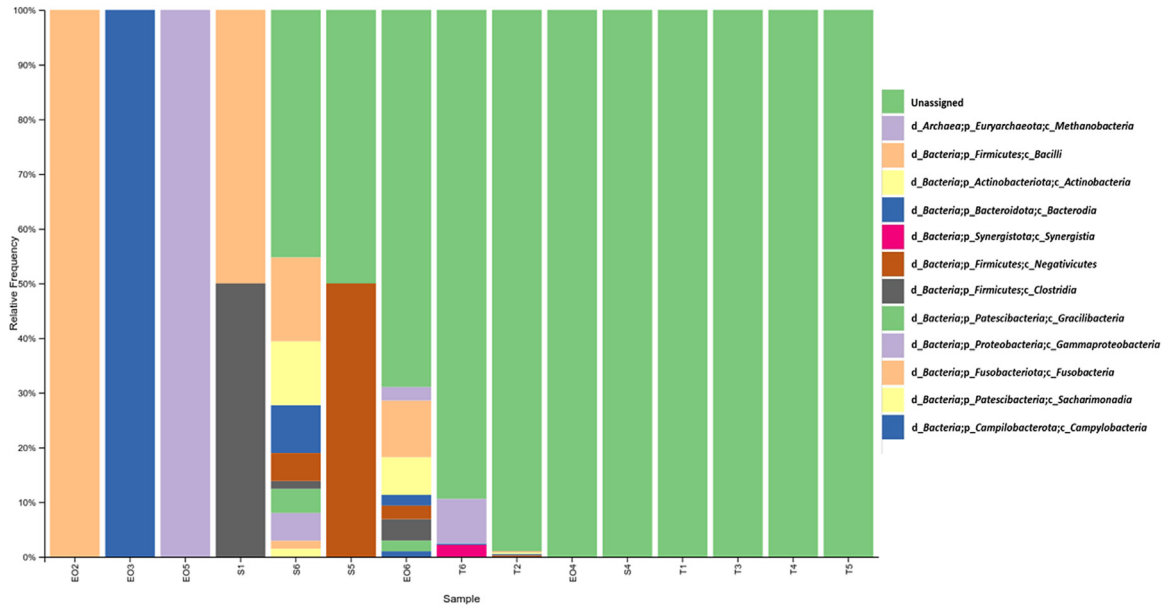

Fig. 3. Stacked bar plots represent the relative abundance at species level for each sample. Three samples were excluded due to DNA concentration insufficiency.

Fig. 4. Phylogenetic analysis. a. Cladogram: Complete phylogenetic tree obtained from all the samples analyzed of the study population. b. Krona diagram of saliva samples at genus level. 44 % of the genetic material could not be assigned to any specific taxa. c. Krona diagram of oral rinse samples at genus level. 70 % of the genetic material could not be assigned to any specific taxa. d. Krona diagram of oral mucosal tissue samples at domain level. 99 % of the genetic material could not be assigned to any specific taxa.

an oral microbiome research study [33]. Our results support the importance of how selecting one sample type or another will reveal very diverse information and so results and conclusions would also substantially differ. Therefore, all studies should consider the relevance of the type of specimen when designing an investigation protocol that aim to study changes in the oral microbiome composition associated with local or systemic pathologies [16,34-36], as well as undesired effects associated with treatments [37].

The characteristics of this study, inherent to its conception and design, imply various limitations, therefore, the results should be taken with caution. We are aware that 6 subjects are a very small sample size. However, our aim is to expand this study in the near

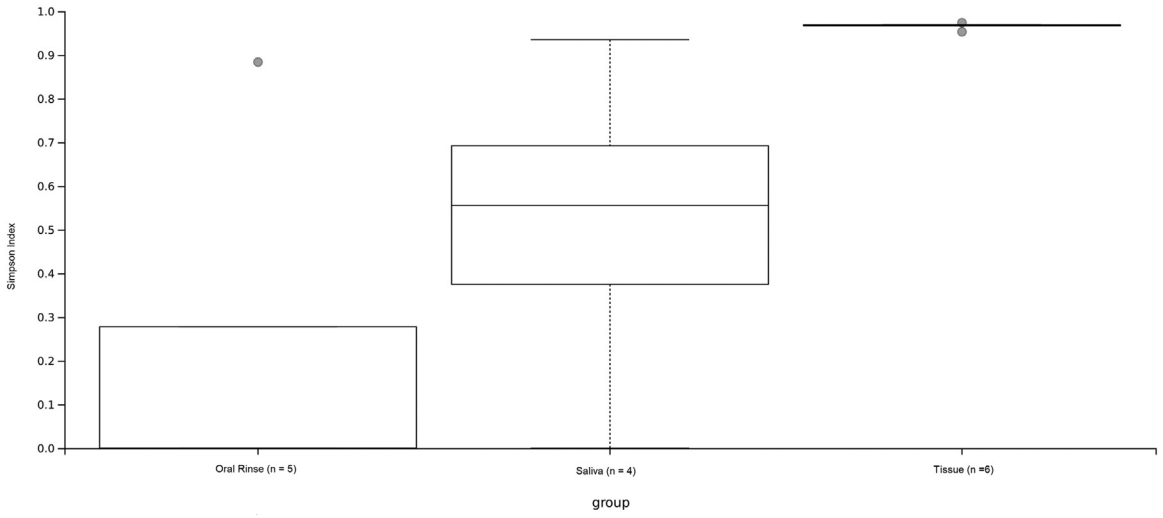

Fig. 5. Alpha diversity analysis: Box plot representing the Simpson's index for each type of sample. Box plots depict median (central horizontal line), 1st quartile and 3rd quartile, and outliers. Statistically significant difference between the alpha diversity of oral rinse and saliva samples (p-value 0,005). Oral rinse showed a superior alpha diversity.

future by recruiting a larger number of participants, so that more reliable conclusions can be drawn. This will also allow us to carry out a statistical analysis of clinical and socio-demographic characteristics of the patients that might be correlated with differences in the populations of microorganisms of the oral flora.

Having to discard 3 samples, for not being able to identify genetic characteristics attributable to bacterial taxonomic units, makes us think that some inaccuracy may have been occurred during samples' processing. Despite the quality analysis performed in terms of the reading depth, misleading in sample handling or treatment may have resulted in the loss of genetic information from specimens that subsequently had to be excluded from the study. Furthermore, these errors may have been accentuated because of the limited experience, given that these are unconventional techniques in a hospital environment due to the lack of clinical usefulness. Also, it is remarkable that there is a high percentage of genetic material that could not be assigned to any known bacterial species among the samples in which genetic features were identified. This fits with the fact that in our results we miss some of the main members of the oral microbiome such as *Streptococcus mitis* species [38] or *Neisseria* and *Haemophilus* genus [15]. This makes us wonder whether there are still technical limitations that may be attributable to an unoptimized or debugged protocol from the commercial companies, the specificity of the reagents and primers [13,18,31], or either a general lack of knowledge about the species that comprise the human oral microbiome [7,36]. The knowledge about the oral microbiome is increasing by leaps and bounds in recent years and, considering that our research was conducted in 2020, we can anticipate some barriers. For instance, recent investigations showed a superiority of V1-V2 sequencing in oral samples versus our method that aimed the V3-V4 region [38]. Besides the limitations of this study, we should also state that the specificity of the technology used in our study is higher than other methods that were traditionally used for bacterial identification such as culturing [7]. Nevertheless, the possibility that our observations were biased due to methodological limitations cannot be excluded.

The need to determine the optimal specimen for the oral microbiome analysis, justifies this research. This pilot study provides a basis for future research on the human oral microbiome. The differences found in the results obtained about microbiome on each sample type support the relevance of choosing an appropriate sample prior to carrying out an investigation of the oral microbiome. Depending on the objectives of the study, one sample should be more appropriate than another as a source of microbial DNA. Thus, oral rinse would be the best option in cases were a wider alpha diversity is needed, whereas whole unstimulated saliva would be more convenient in situations where a larger amount of bacterial genetic material is required.

Ethics approval statement

The present research was approved by the Galician Ethics Committee of Clinical Research (Ref. No. 2020/552) and conducted in accordance with the guidelines for Good Clinical Practice and the Declaration of Helsinki. All participants signed the informed consent form.

Financial interests

The authors declare they have no financial interests.

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Data availability

The data that support the findings of this study are not openly available due to reasons of sensitivity and are available from the corresponding author upon reasonable request. Data are located in controlled access data storage in the Microbiology Unit of the Complexo Hospitalario Universitario de Santiago de Compostela.

Declaration of competing interest

None

CRediT authorship contribution statement

Manuel Eros Rodríguez-Fuentes: Conceptualization, Data curation, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Mario Pérez-Sayáns:** Conceptualization, Investigation, Methodology, Project administration, Resources, Writing –

review & editing. **Gema Barbeito-Castiñeiras:** Methodology, Validation, Writing – review & editing. **Alberto Molares-Vila:** Data curation, Formal analysis, Methodology, Software, Visualization, Funding acquisition. **Irene B. Prado-Pena:** Conceptualization, Methodology. **Gisela C.V. Camolesi:** Data curation, Investigation, Methodology. **Rafael López-López:** Conceptualization, Funding acquisition, Resources, Supervision.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jormas.2024.101991.

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