Heliyon 9 (2023) e20303

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Difference in microbiome compositions of healthy peri-implant sulcus and peri-implantitis sulcus from the same patient

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ARTICLE INFO

Keywords:
Microbiome composition
Healthy peri-implant sulcus
Peri-implantitis sulcus
rRNA sequencing

ABSTRACT

Objective: The objective of this study is to compare the microbiome of healthy peri-implant sulcus (C) and peri-implantitis sulcus (U) from the same patient and analyze the difference in the microbiome composition.

Materials and methods: DNA samples of subgingival biofilms from 10 C (control group) and 10 U (uncontrolled group) sites were sent to Microbiome Center in Korea Research Institute of Biomedical Science and analyzed using 16s rRNA gene amplification and sequencing (MiSeq, Illumina) and human oral microbiome database (HOMD).

Results: At the phylum level, *Firmicutes* and *Proteobacteria* were more abundant in group C, while *Firmicutes* and *Bacteroidetes* were dominant in group U. At the genus level, the core peri-implant microbiome was *Streptococcus* in group C. On the other hand, the core peri-implant microbiome was *Porphyromonas,* especially *P. gingivalis* in group U.

Conclusion: In this study, the microbiome composition of peri-implantitis sulcus was different from that of healthy peri-implant sulcus from the same patient. The peri-implantitis microbiome was pathogen-enriched and was similar to the microbiome associated with periodontitis.

1. Introduction

Dental implant installment has become a popular treatment method for rehabilitating edentulous patients. Despite its high success rate, implant treatment has complications, such as peri-implantitis, a bacterial biofilm-associated pathological condition characterized by inflammation and bone loss [1]. Bacteria associated with this destructive disease are part of the normal oral microbiota [2] since bacteria from saliva and supragingival plaque on the implant surface colonize peri-implant sulcus [3]. The microbiome of healthy peri-implant sulcus is characterized by a low ratio of anaerobic to aerobic species and few periodontal pathogens [4]. However, under certain ecological shifts, bacteria associated with inflammation become dominant and pathogenic, acting in concert [2].

In order to understand and treat peri-implantitis, it is important to analyze changes in the peri-implant microbiome caused by the ecological shift. In this study, one of the culture-independent methods, 16s ribosomal RNA (rRNA) gene sequencing was used to characterize the peri-implant microbiome. Unlike other close-ended molecular approaches, 16s rRNA Illumina sequencing identifies untargeted but potentially relevant taxa by an open-ended characterization of the microbiome under study [5]. Thus, this method can identify high levels of microbial diversity. In addition, 16s rRNA gene sequencing is cost-effective since higher sequence quality can be obtained at a much lower cost per sequence [5]. The detailed analysis of the peri-implant microbiome helps not only understand the etiology of peri-implantitis but also develop individualized and targeted treatment strategies, which are more effective than generic

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https://doi.org/10.1016/j.heliyon.2023.e20303
Received 5 March 2023; Received in revised form 2 September 2023; Accepted 18 September 2023
Available online 20 September 2023

treatments such as mechanical debridement and antiseptic application [6].

The purpose of this study was to compare the microbiome compositions of healthy peri-implant sulcus and peri-implantitis sulcus from the same patient using 16s rRNA gene sequencing and analyze changes in the peri-implant microbiome.

2. Materials and Methods

2.1. Patient selection

This study was approved by the Institutional Review Board of the Catholic University of Korea, Uijeongbu St. Mary's Hospital (UC18OESI0147) and funded by the Catholic University of Korea, Uijeongbu Institute for Clinical Medicine. The study was in compliance with STROBE guideline. Eleven study subjects were selected according to the following criteria - patients who had at least

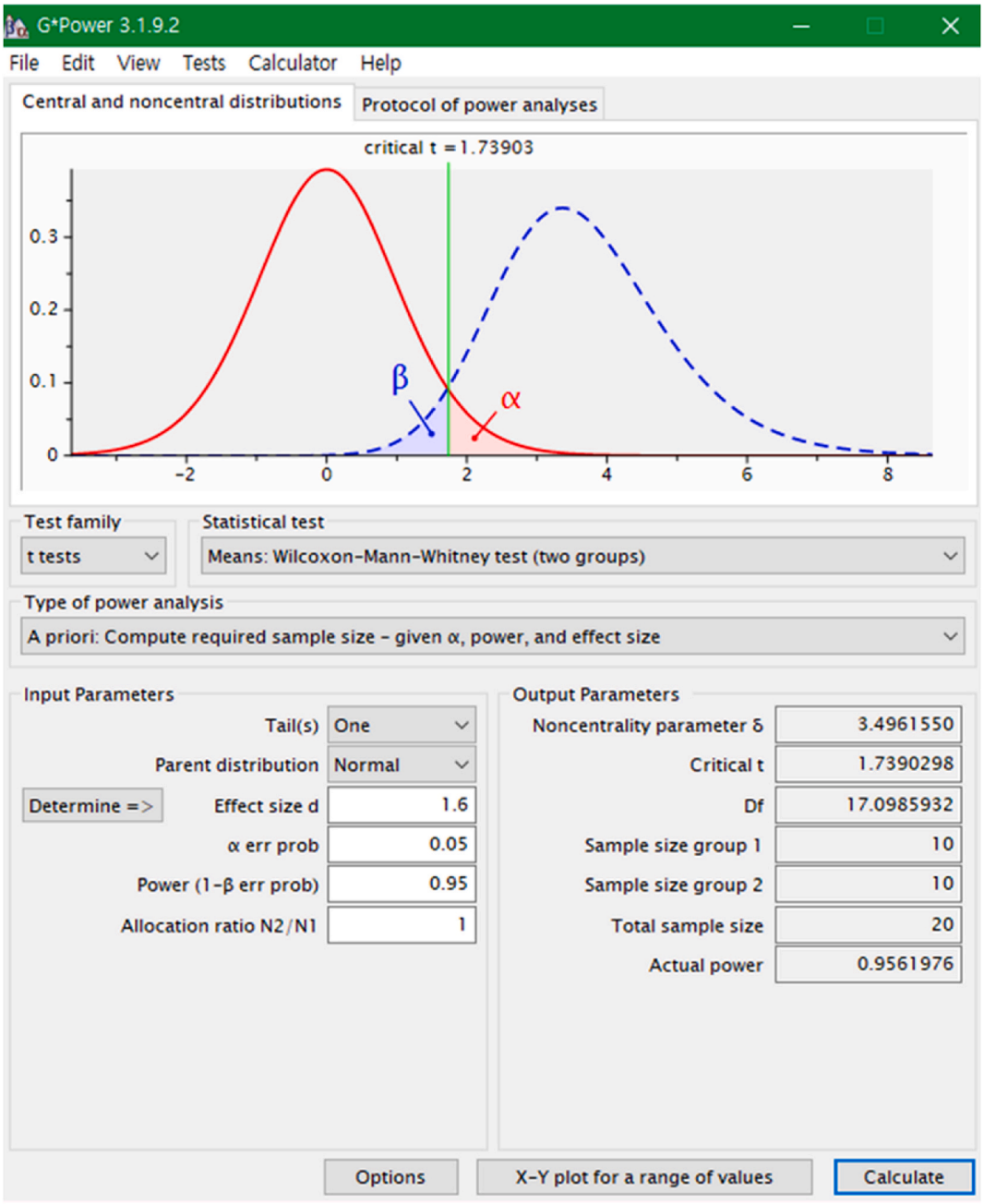

Fig. 1. Sample size and power calculations using G power.

one 1-year checkup appointment after placing implant fixtures and installing fixed prostheses, patients who had at least one healthy implant and one peri-implantitis implant in posterior regions, healthy male/female adults aged under 80, and patients who understood the research objective and provided informed consent. Exclusion criteria were (i) pregnancy, (ii) diabetes, (iii) HIV infection, (iv) currently taking systemic immunosuppressant medications, (v) currently taking any bisphosphonate, and (vi) antibiotic therapy within 3 months before sampling.

In this study, the taxa count distributions between sample groups were compared. Distance matrices identifying the beta diversity between samples were analyzed, and the distribution of distances within groups to those between groups was then compared. Sample size and power calculations for the design of this study were performed using data from the pilot study to obtain appropriate intra- and between-group distance distributions (Fig. 1).

2.2. Observation categories and measurement instruments

History taking of patients was done to check antibiotic use within the past 3 months and systemic health condition. In a clinical appointment, the pocket depths of six sites (mesio-, mid-, disto-buccal and mesio-, mid-, disto-lingual) at selected implants were measured using sterilized perio probes. Sterilized endodontic paper points were placed in four sites (mesial, distal, buccal, lingual) of the peri-implant sulcus to obtain subgingival plaque. This sampling technique has been an internationally popular method for microbial culture studies [7]. The subgingival plaque was acquired from healthy peri-implant sulcus and diseased peri-implantitis sulcus of the same patients.

Healthy peri-implant sulcus showed no local infection, swelling, fistula, BOP, or peri-implant mucositis. Implants with peri-implantitis were selected based on the definitions presented in the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions [8]. In the clinical setting, bleeding on probing was used to detect soft tissue inflammation, and progressive bone loss was identified by the comparison of periapical radiographs taken after implant prosthesis installation and checkup appointments. BOP, increase in probing depth of peri-implant sites, and radiographic bone loss were used to determine case definitions for peri-implantitis.

In this study, the definitions of per-implantitis were as follows: bleeding on probing with or without suppuration, mean probing depth of 5–6 mm or more, bone loss higher than 3–4 mm, and configurations of intrabony (Class I) defect or suprabony(Class II) defect or both combined on periapical radiographs.

2.3. Research methods

In a dental clinic at the Catholic University of Korea, Uijeongbu St. Mary's Hospital, the subgingival plaque was sampled from healthy and diseased peri-implant sulcus of patients who had more than one implant with peri-implantitis among implant prostheses under more than a year of functional loading. Supragingival plaque of selected implants was removed using sterilized gauze, and subgingival plaque was sampled from four sites (mesial, distal, buccal, lingual) of the implant sulcus by placing sterilized paper points for 10 s. Acquired samples were put in cryo tubes, which were then stored in a liquid nitrogen tank. The stored samples were sent to the Korea Research Institute of Biomedical Science for DNA extraction and analysis (Fig. 2) [9]. Research bias was avoided since DNA analysis of the collected data was done by the independent research institute.

16s rRNA gene fragments were analyzed using synthesis chemistry for bi-directional amplicon sequencing by MiSeq Desktop Sequencer; Reagent Kit v2; Illumina [10]. PCR reactions were done independently, and the V5–V7 hypervariable region was amplified with barcoded primers 785F (F 50-GGATTAGATACCCBRGTAGTC-30) and 1175R (R 50-ACGTCRTCCCCDCCTTCCTC-30) [11]. The acquired data were compared with the human oral microbiome database (HOMD) to conclude the constituents and their proportion in peri-implant biofilm.

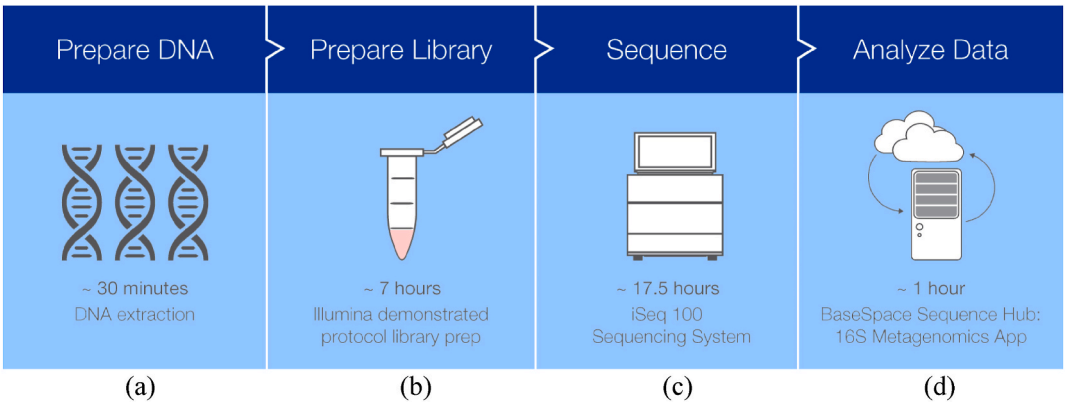

Fig. 2. 16s metagenomics sequencing workflow: (a) Prepare DNA for 30 min, (b) Prepare library for 7 h, (c) Sequencing for 17.5 h, and (d) Analyzing data for 1 h (adapted from Illumina [9]).

2.4. Statistical analysis

Analysis of the composition of microbiomes (ANCOM) was used to compare microbiomes from 10 healthy(C) and 10 unhealthy peri-implantitis(U) sulcus. Nonparametric two-sample t-tests using Monte-Carlo permutations compared alpha diversity measures of 10 C and 10 U sites. Nonparametric significance tests, permutational multivariate analysis of variance (PERMANOVA), and analysis of similarities (ANOSIM) based on Bray-Curtis dissimilarity coefficients were used to analyze differential taxonomic groups between C and U sites. The relative abundance of the top hits was analyzed using the pairwise Wilcoxon signed-rank test (significant at $p < 0.05$ after Bonferroni's correction).

3. Results

3.1. Clinical outcomes

One of 11 patients originally enrolled in the study dropped out, so 10 samples were collected from healthy (C) and peri-implantitis (U) implant sulcus of 10 patients. Healthy peri-implant sulcus showed no local infection, swelling, fistula, BOP, or peri-implant mucositis. The mean values of probing depth were less than 4 mm. Peri-implantitis sites showed BOP and mean probing depth values of ≥5–6 mm (Table 1). Also, bone loss was higher than 3–4 mm in some or all sites. In addition, configurations of intrabony defect, suprabony defect, or both combined were observed on periapical radiographs of peri-implantitis sites.

3.2. Analysis of alpha diversity

10 samples from healthy (C) and peri-implantitis (U) sulcus of 10 patients were analyzed. Target read count, Shannon species diversity, and Good's coverage of library were similar between group C and U (Table 2).

3.3. Analysis of community diversity

3.3.1. Phylum level

At the phylum level, when the samples of healthy (C) peri-implant sulcus and unhealthy (U) peri-implantitis sulcus were compared, the composition of *Bacteriodetes* increased in group U except subject 2. Also, the composition of *Proteobacteria* decreased in group U except for subject 2 and 7 (Fig. 3).

Mean relative abundance was compared. Group C was mainly composed of *Firmicutes* (45.2%) and then *Proteobacteria,* whereas group U consisted of *Firmicutes* (32.5%) and then *Bacteroidetes* (27.9%) (Fig. 4).

3.3.2. Genus level

At the phylum level, when the samples of group C and U were compared, the composition of *Streptococcus* decreased in group U, and that of *Porphyromonas* increased in group U for all subjects (Fig. 5).

Mean relative abundance was compared. At the genus level, the microbiome of group C was mainly composed of *Streptococcus* (21.2%), while the microbiome of group U consisted of *Streptococcus* only by 7.9%. 4.9% of group C was *Neisseria* genus, whereas only 1.0% of group U was *Neisseria* genus. On the contrary, the microbiome of group U was mainly composed of *Porphyromonas* (13.2%), specifically *Porphyromonas gingivalis* (10.4%). (Fig. 6).

4. Discussion

In this study, the microbiome of healthy (C) peri-implant sulcus constitutes *Firmicutes* and *Proteobacteria* at the phylum level and *Streptococcus* at the genus level. The microbiome of unhealthy (U) peri-implantitis sulcus is composed of *Firmicutes* and *Bacteroidetes* at

Table 1
Sample characterization and clinical outcomes from healthy (C) and peri-implantitis (U) sulcus.

Sample ID	#1	#2	#3	#4	#5	#6	#7	#8	#9	#11
Gender	F	M	M	M	F	M	M	F	F	M
Age	70	63	60	62	65	57	59	63	59	77
Sampling sites (healthy sulcus)	#47i (1_C)	#14i (2_C)	#26i (3_C)	#27i (4_C)	#36i (5_C)	#34i (6_C)	#37i (7_C)	#16i (8_C)	#46i (9_C)	#36i (11_C)
Mean PD±SD (mm)	2.25 ±0.5	2.25 ±0.5	2.25 ±0.5	2.75 ±0.96	1.75 ±0.5	3.75 ±0.5	4 ±0	2 ±0	1.5 ±0.58	2 ±0
Sampling sites (peri-implantitis)	#16i (1_U)	#46i (2_U)	#17i (3_U)	#37i (4_U)	#46i (5_U)	#45i (6_U)	#26i (7_U)	#36i (8_U)	#36i (9_U)	#45i (11_U)
Mean PD±SD (mm)	6 ±0.82	6.5 ±0.58	6 ±0.82	7 ±2	6 ±1.2	7.25 ±0.96	6.5 ±1	6 ±0	6.67 ±1.2	8 ±0.82
Mean bone loss±SD (mm)	2.45 ±0.78	7.15 ±0.07	3.15 ±0.49	7.3 ±1.56	6.3 ±0.99	6.35 ±0.64	2.55 ±0.78	5.9 ±1.98	5.15. ±0.78	6.1 ±0.42
Bone loss class	I	I	II	III	III	I	I	III	III	III

Table 2
Alpha diversity. 1) Number read count: the number of nucleotides sequenced by Next Generation sequencing. 2) Shannon species diversity: microorganism diversity index. 3) Good's coverage of library: detection ratio of microorganisms in a sample.

No.	Sample ID	Target Reads count	Shannon Species Diversity	Good's coverage of library(%)
1	1_C	85,838	4.09	99.94
2	2_C	86,739	3.98	99.90
3	3_C	85,816	3.59	99.82
4	4_C	86,493	4.29	99.92
5	5_C	82,906	3.16	99.83
6	6_C	85,473	4.18	99.89
7	7_C	85,269	3.46	99.87
8	8_C	73,837	4.72	99.74
9	9_C	85,332	4.02	99.88
10	11_C	87,096	3.16	99.85
11	1_U	84,086	4.00	99.90
12	2_U	87,010	4.22	99.85
13	3_U	87,742	3.45	99.90
14	4_U	87,078	4.30	99.86
15	5_U	86,093	3.29	99.85
16	6_U	87,986	3.58	99.84
17	7_U	52,248	2.88	99.93
18	8_U	84,441	3.58	99.93
19	9_U	88,099	4.34	99.91
20	11_U	86,967	4.40	99.91

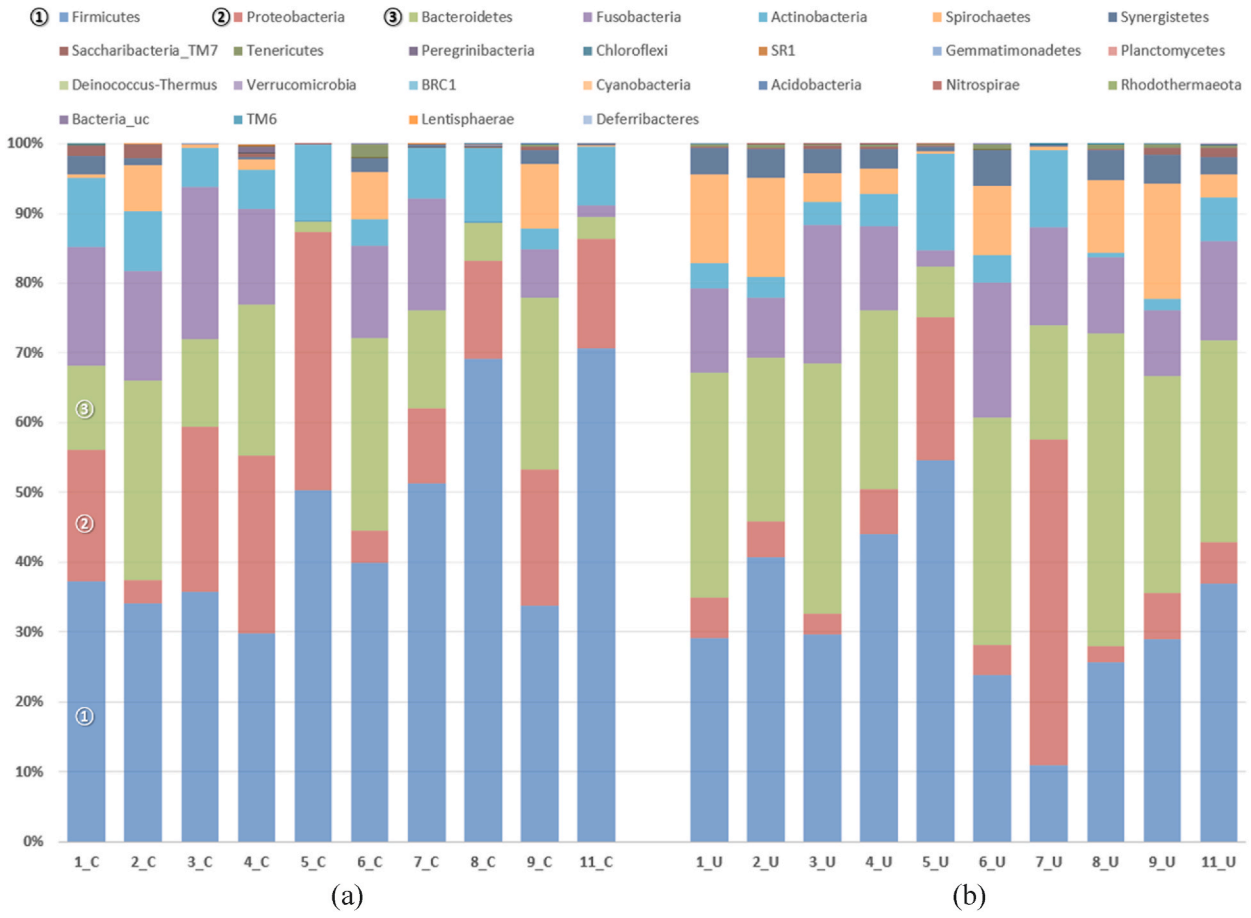

Fig. 3. Sample comparison at the phylum level: (a) C group – healthy peri-implant sulcus and (b) U group – unhealthy peri-implantitis sulcus.

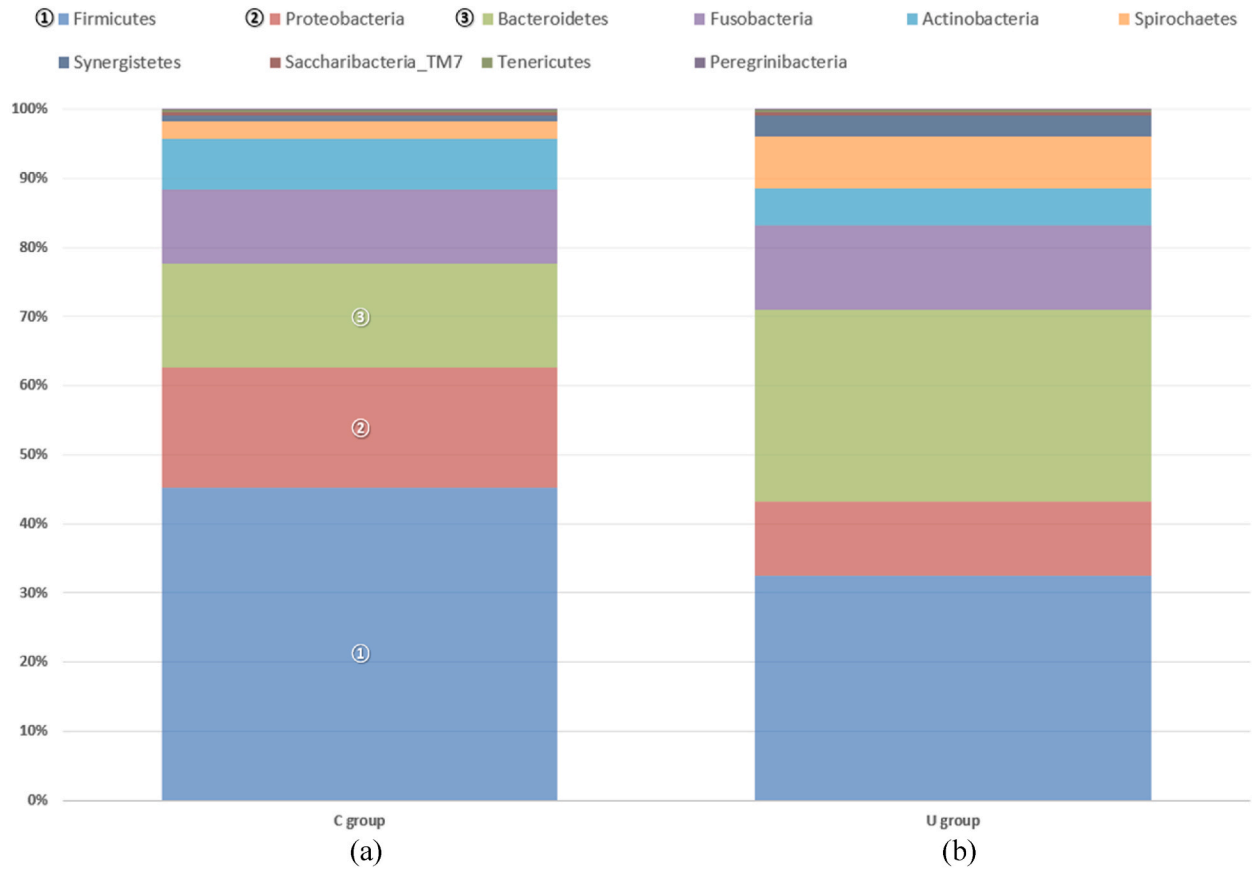

Fig. 4. Mean relative abundance at the phylum level: (a) C group – healthy peri-implant sulcus and (b) U group – unhealthy peri-implantitis sulcus.

the phylum level and *Porphyromonas*, especially *P. gingivalis* at the genus level. The study by Sanz-Martin et al. reported that healthy peri-implant sites were colonized by *Proteobacteria* and *Actinobacteria* phyla. Also, *Streptococcus* (phylum *Firmicutes*) and *Neisseria* were abundant in healthy peri-implant sites [5]. On the other hand, peri-implantitis sites harbored genera *Porphyromonas* (phylum *Bacteroidetes*), *Treponema* (phylum *Spirochetes*), and *Filifactor* (phylum *Firmicutes*). In addition, these diseased sites contained higher levels of classic pathogens, the so-called 'red complex' (*Porphyromonas gingivalis, Tannerella forsythia, Treponema denticola*). The results of this study align with previous findings by Sanz-Martin et al. [5].

The microbiome of healthy peri-implant sites mainly consists of Gram-positive cocci, non-motile bacilli, and few Gram-negative anaerobic species [12]. As inflammation progresses, bone loss occurs, and the peri-implant pocket deepens. Peri-implantitis is characterized by bleeding on probing and deep peri-implant pocket, which causes the ecological shifts favoring anaerobic bacteria over aerobic species due to low oxygen condition. In most cases, the composition of the peri-implantitis microbiome is similar to that of the periodontitis microbiome, dominated by Gram-negative bacteria. Gram-negative, black-pigmented, motile, and anaerobic species are commonly found in deep periodontal pockets [13]. The majority of the peri-implantitis 'checkerboard' studies based on the traditional culture studies of periodontitis sites confirmed that the peri-implant pocket shares a similar microbial profile with the periodontal pocket. The cluster of red complex (*P. gingivalis, T. forsythia, T. denticola*) inhabited peri-implantitis sites more abundantly than healthy sites [14]. The change of bacterial profile from group C to group U in this study supports these previous findings.

This study sampled biofilms from healthy peri-implant and diseased peri-implantitis sites of the same patient. It was reported that microbiome compositions of peri-implant submucosa differed greatly among individuals, and these inter-subject variations even outweighed differences between healthy and peri-implantitis sites [15]. Therefore, comparing the different groups of samples from the same individuals can be crucial to eliminate the effects of confounding factors caused by individual differences [16]. For this advantage, the studies done by Song et al. and Ganesan et al. compared pairs of healthy and diseased implants from the same patients to analyze differences in microbiome and gene expression [16,17]. Thus, it is evident that in this study, the difference in bacterial compositions of healthy peri-implant and peri-implantitis sulcus is due to the ecological shift favoring anaerobes over aerobes caused by the deep peri-implant sulcus and not due to host difference.

This study has limitations in that it has a small sample size and only compares the microbiome compositions of healthy and peri-implantitis sulcus. In further studies, sample sizes should be larger to approximate the population more closely. Also, it will be more informative to include the microbiome composition of a transitional stage from healthy to peri-implantitis, such as peri-implant mucositis. In addition to that, it will be interesting to compare the microbiome compositions of healthy and diseased periodontal

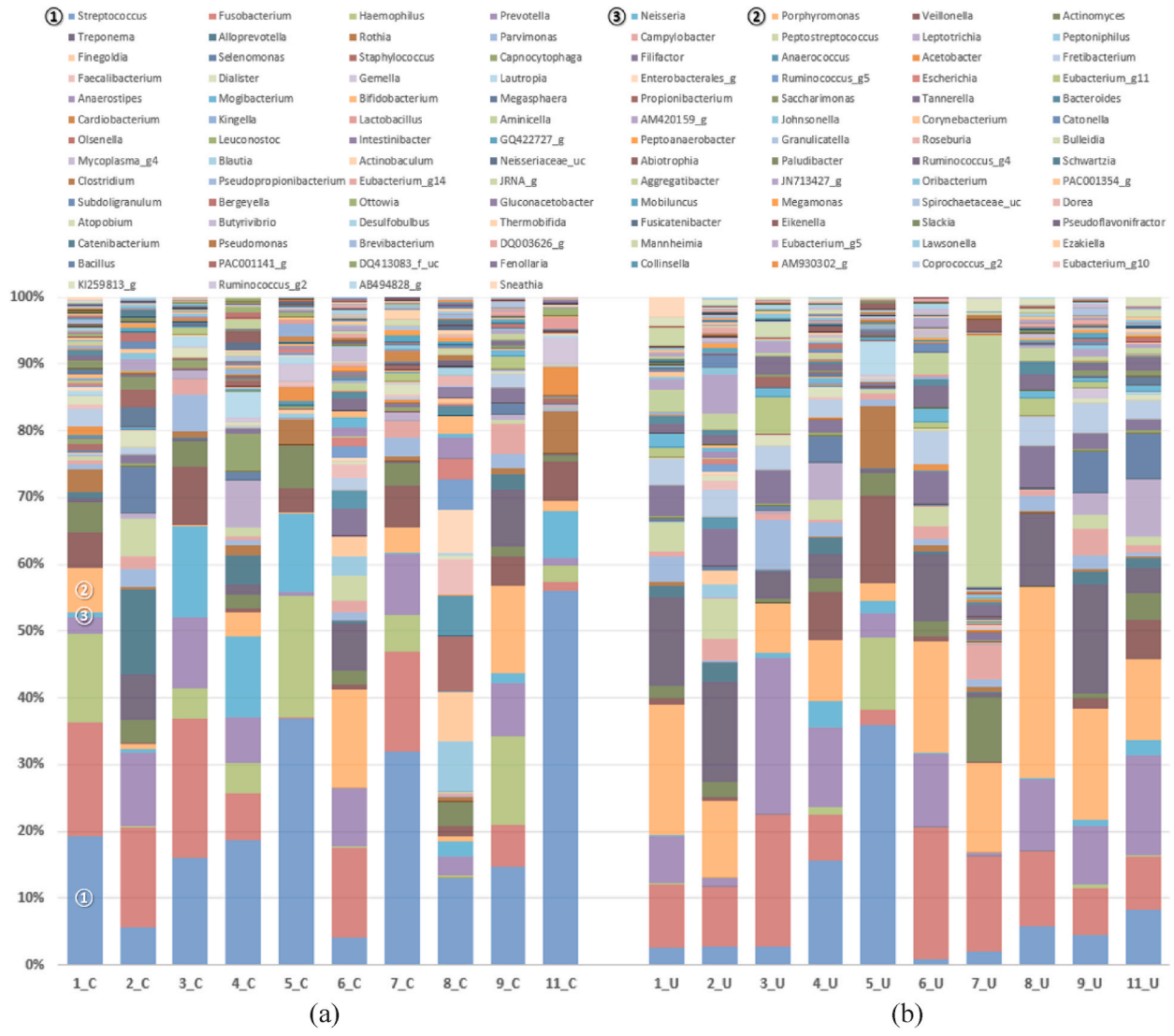

Fig. 5. Sample comparison at the phylum level: (a) C group – healthy peri-implant sulcus and (b) U group – unhealthy peri-implantitis sulcus.

pockets and healthy peri-implant and diseased peri-implantitis sites from the same patient.

5. Conclusion

In this study, *Firmicutes* and *Proteobacteria* phyla and *Streptococcus* genus colonized healthy peri-implant sulcus, whereas *Firmicutes* and *Bacteroidetes* phyla and *Porphyromonas*, especially *P. gingivalis* genus are abundant in unhealthy peri-implantitis sulcus. In conclusion, the microbiome composition of unhealthy peri-implantitis sulcus was different from that of healthy peri-implant sulcus from the same patient. The peri-implantitis microbiome was anaerobe and pathogen-enriched and was similar to the microbiome associated with periodontitis.

Author contribution statements

Hyun Jung Jung: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper. Won Lee: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

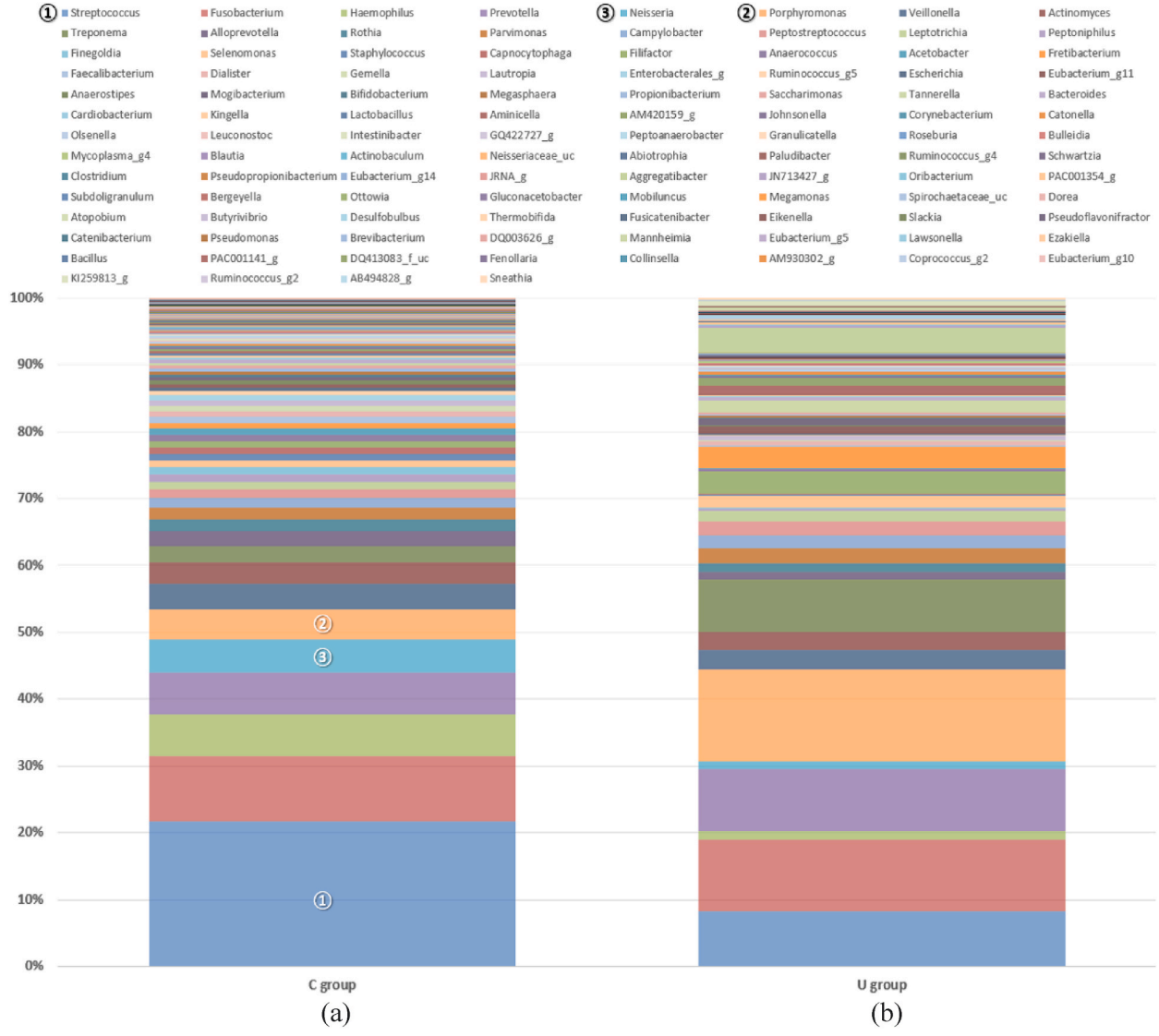

Fig. 6. Mean relative abundance at the phylum level: (a) C group – healthy peri-implant sulcus and (b) U group – unhealthy peri-implantitis sulcus.

Data availability statement

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors wish to acknowledge the financial support of The Catholic University of Korea Uijeongbu St. Mary's Hospital Clinical Research Laboratory Foundation made in the program year of 2018.

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