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Research Article
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Microbial profiling of peri-implantitis compared to the periodontal microbiota in health and disease using 16S rRNA sequencing

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ABSTRACT

Purpose: The objective of this study was to analyze the microbial profile of individuals with peri-implantitis (PI) compared to those of periodontally healthy (PH) subjects and periodontitis (PT) subjects using Illumina sequencing.
Methods: Buccal, supragingival, and subgingival plaque samples were collected from 109 subjects (PH: 30, PT: 49, and PI: 30). The V3-V4 region of 16S rRNA was sequenced and analyzed to profile the plaque microbiota.
Results: Microbial community diversity in the PI group was higher than in the other groups, and the 3 groups showed significantly separated clusters in the buccal samples. The PI group showed different patterns of relative abundance from those in the PH and PT groups depending on the sampling site at both genus and phylum levels. In all samples, some bacterial species presented considerably higher relative abundances in the PI group than in the PH and PT groups, including *Anaerotignum lactatifermentans*, *Bacteroides vulgatus*, *Faecalibacterium prausnitzii*, *Olsenella uli*, *Parasutterella excrementihominis*, *Prevotella buccae*, *Pseudoramibacter alactolyticus*, *Treponema parvum*, and *Slackia exigua*. Network analysis identified that several well-known periodontal pathogens and newly recognized bacteria were closely correlated with each other.
Conclusions: The composition of the microbiota was considerably different in PI subjects compared to PH and PT subjects, and these results could shed light on the mechanisms involved in the development of PI.

Keywords: Dental plaque; High-throughput nucleotide sequencing; Microbiota; Peri-implantitis; Periodontitis

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Conflict of Interest
No potential conflict of interest relevant to this article was reported.

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INTRODUCTION

Advances in the surgical and prosthodontic techniques of dental implants have led to a survival rate of more than 96% in implants that function for a long period [1]. However, as many implants are placed in patients every year, the prevalence of peri-implant disease has gradually increased and has recently been reported to be 45% at the patient level [2]. Peri-implantitis (PI) is a progressive peri-implant disease that involves an inflammatory lesion in the peri-implant mucosa with additional loss of the supporting bone after initial bone remodeling [3].

Poor oral hygiene, a history of severe periodontitis (PT), and no regular maintenance care have been suggested as risk indicators for PI [4]. In particular, bacteria in the biofilm of teeth or implants produce toxins that cause tissue damage and further intensify the host's inflammatory response, eventually leading to the destruction of the surrounding tissue [5,6]. Both PT and PI are inflammatory diseases associated with oral polymicrobial infection [7]. However, the characteristics of the tissues surrounding implants present distinct differences compared to that of the tooth, including an absence of the periodontal ligament, a poor vascular system, and the arrangement of the connective tissues [8]. Due to these differences, PI is likely to worsen and easily spread to supporting bone [8,9]. Understanding the characteristics of the microbiota associated with PI may help to develop therapeutic methods and effective prevention specific to PI.

The microbiota associated with biofilms surrounding healthy implants have been reported to be similar to those surrounding healthy teeth [10,11]. Studies published in the early 2000s found that the microbial composition associated with peri-implant disease was similar to that of chronic PT [10,12]. Most of these studies used conventional methods such as culture techniques, microscopy, and the Sanger method. Recently, next-generation sequencing (NGS) has been introduced. NGS can perform multiple reactions at the same time, carry out sequencing with a small amount of samples, and detect non-culturable organisms; it has also revolutionized sequencing in terms of the read length, accuracy, time, and cost [13]. NGS is used in various fields of dentistry, such as research on periodontal disease-related microorganisms [14], the analysis of mutations of cancer-related genes in oral squamous cell carcinoma [15], and the analysis of salivary microbiota related to Sjögren syndrome [16]. In particular, Illumina sequencing has been a widely used NGS platform. The advantages of this platform are a lower cost per sequence, a lower per-base error rate, and a greater number of reads with a higher average quality than that of other platforms [13].

The objective of this study was to analyze the microbial profile of subjects with PI compared to that of periodontally healthy (PH) subjects and subjects with PT using Illumina sequencing.

MATERIALS AND METHODS

Study population and clinical examination

Plaque samples were obtained from PH subjects (n=30), PT subjects (n=49), and PI subjects (n=30) at the Department of Periodontology, Pusan National University Dental Hospital, Yangsan, Republic of Korea. The sampling period was from March 2018 to August 2018. The inclusion criteria were as follows: (a) the presence of 20 or more teeth, (b) the absence of systemic diseases that may affect periodontal status as evidenced by the patient's medical

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history, (c) no periodontal treatment within the last 3 months, and (d) no history of systemic antibiotic or anti-inflammatory drug use within the past 6 months. The criteria for exclusion were as follows: (a) pregnant or breastfeeding, (b) acute infection or chronic mucosal lesion of the oral cavity, and (c) heavy smoking (>20 cigarettes/day).

Full-mouth clinical examinations were carried out by 1 periodontist to characterize the periodontal and peri-implant status. These examinations evaluated the probing pocket depth (PPD), clinical attachment level (CAL), plaque index, and gingival index (GI). The diagnosis of diseases followed guidelines presented by the 2017 World Workshop on the Classification of Periodontal and Peri-implant Diseases and Conditions [17]. Periodontal health was diagnosed when subjects exhibited PPD ≤3 mm, bleeding on probing ≤10%, no clinical attachment loss, and no radiological bone loss. PT was diagnosed as follows: 1) interdental clinical attachment loss is detectable at ≥2 non-adjacent teeth, or 2) buccal of oral clinical attachment loss ≥3 mm with PPD >3 mm is detectable at ≥2 teeth. PI was diagnosed in subjects who had an implant with radiographic evidence of marginal bone loss ≥3 mm and/or PPD ≥6 mm in conjunction with profuse bleeding. The CAL of the implant was calculated as the distance from a fixed landmark (implant-abutment junction) to the bottom of the implant sulcus/pocket [18]. Each subject was assigned to only 1 group, and there were no diseased implants in either the PH or PT group. Written informed consent was obtained from all participants prior to conducting the study, and the experimental protocol was approved by the Institutional Review Board (IRB) of Pusan National University Dental Hospital (IRB No. PNUDH-2017-023).

Plaque sample collection

Plaque samples were obtained during the full-mouth periodontal examination. Buccal and supragingival samples were collected from the PH, PT, and PI subjects, while subgingival samples were only collected from the PT and PI subjects. The buccal samples were collected from the mucosa of both cheeks using a sterile micro-brush and were placed in a separate sterile 1.5-mL microcentrifuge tube. Before collecting the supragingival samples, the site was dried with a cotton roll to isolate the sample from any saliva or blood. The subgingival samples were acquired from the deepest periodontal pocket and peri-implant site using a sterile Gracey curette, after which the samples were placed in the tube mentioned above. All the samples were collected and stored at −80°C for subsequent processing.

Extraction of total genomic DNA

Total DNA extraction from the samples was performed using a Gram-positive DNA purification kit (Lucigen, Middleton, WI, USA) following the manufacturer's instructions. The final concentration measurements were conducted with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and the samples were stored at −80°C until use.

Polymerase chain reaction amplification and sequencing analysis

The V3-V4 region of the 16S ribosomal RNA gene was amplified by polymerase chain reaction, and the purified amplicons were pooled in an equimolar and paired-end sequence (2×300) on a MiSeq platform (Illumina, San Diego, CA, USA). The raw FastQ files were demultiplexed and processed using the tools available in QIIME2 (version 2019.7) [19]. The taxonomy classification was performed with a naïve Bayes machine-learning classifier using representative sequence sets for each dada2 sequence variant. The Human Oral Microbiome Database (HOMD, v15.2) [20] was used as the reference database.

Bioinformatic analysis, statistical analysis, and visualization

Several normality tests were performed for each set of data. The mean clinical parameters were compared using 1-way analysis of variance (ANOVA) followed by the *post hoc* Games-Howell test with $P<0.05$ as the statistical significance level. The operational taxonomic unit table was rarefied, and then alpha diversity was evaluated by 2 metrics: the species richness was estimated using the Chao1 index, and the Shannon index was used to estimate the evenness of the sample microbiota. The Mann-Whitney *U* test was performed to evaluate the significance of differences in the alpha diversity indices among the groups ($P<0.05$). The unweighted UniFrac distance was used to evaluate the similarities of microbial community composition among all samples, and the overall structure was visualized with a principal coordinate analysis (PCoA) plot. The non-parametric permutational ANOVA test was used to analyze group differences with 1,000 permutations [21]. The linear discriminant analysis effect size with default settings was used to identify taxa whose abundances were significantly different among the groups [22]. All the PCoA analyses, scatter diagrams, and box plots were conducted using R (R Foundation, Vienna, Austria).

Network analysis

To visualize interactions and characterize the microbial community, Sparse Correlations for Compositional Data (SparCC) [23] was used to calculate the Spearman correlation coefficient with the corresponding *P* value between each pair of genera. The network was then visualized using Cytoscape [24].

RESULTS

The clinical characteristics of the PH, PT, and PI subjects are listed in **Table 1**. Compared with the PH group, the mean values of PPD, CAL, GI, and the plaque index in the PT and PI groups showed significant differences ($P<0.01$). The mean values of both GI and the plaque index were significantly higher in the PT group than in the PI group ($P<0.05$).

Microbial community diversity

The Chao1 index showed that the species richness in all samples was significantly higher in the PI group than in the other groups ($P<0.01$). The Shannon index showed that the evenness of the sample microbiota in both the PI and PT groups was significantly higher than that in the PH groups in the supragingival samples ($P<0.01$), but no significant difference was found in the other samples (**Figure 1A**).

Table 1. Characteristics of the subjects

Parameters	PH (n=30)	PT (n=49)	PI (n=30)	*P* value		
				PH-PT	PH-PI	PT-PI
Patient age (yr)	26.9±5.7	50.7±9.8	55.8±10.1			
Sex						
Male	15 (50.0)	36 (73.5)	15 (50.0)			
Female	15 (50.0)	13 (26.5)	15 (50.0)			
PPD (mm; average of all sites)	2.4±0.04	3.5±0.8	3.2±0.4	<0.01	<0.01	0.19
PPD (mm; subgingival sampled site)	N/A	7.7±3.1	7.8±2.7	N/A	N/A	0.98
CAL (mm)	2.4±0.04	3.9±1.2	3.7±0.7	<0.01	<0.01	0.54
GI	0.1±0.01	0.9±0.3	0.6±0.2	<0.01	<0.01	<0.05
Plaque index	0.1±0.01	0.5±0.06	0.4±0.03	<0.01	<0.01	<0.01

Values are presented as mean±standard deviation or number (%). $P<0.05$, analysis of variance with the *post hoc* Games-Howell test.
PPD: periodontal pocket depth, CAL: clinical attachment level, GI: gingival index, PH: periodontally healthy, PT: periodontitis, PI: peri-implantitis, N/A: not available.

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Figure 1. Alpha and beta diversities of microbial communities grouped by disease status and sampling sites. (A) Alpha diversity values in the buccal, supragingival, and subgingival samples were calculated by the Chao1 index and Shannon index. (B-D) Beta diversity values in the buccal, supragingival, and subgingival samples, respectively.
PH: periodontally healthy, PI: peri-implantitis, PT: periodontitis.
[a]$P<0.05$; [b]$P<0.01$.

Figure 1B showed a clear distinction and significant difference among the microbial clusters of the PI, PT, and PH groups in the buccal samples ($P<0.01$). In the supragingival and subgingival samples, the microbial clustering was not significant (**Figure 1C and D**).

Relative abundance of microbial composition

At the phylum level, the 7 most abundant phyla were *Firmicutes*, *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Fusobacteria*, *Spirochaetes*, and *Synergistetes* (**Figure 2A**). The relative abundances of *Firmicutes*, *Spirochaetes*, and *Synergistetes* in the diseased groups (PT and PI) were higher than those in the healthy group (PH) for both the buccal and supragingival samples. When comparing the diseased groups, *Firmicutes* and *Actinobacteria* showed higher relative abundances in the PI group than in the PT group in the buccal samples. In the subgingival samples, the relative abundances of *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, and *Fusobacteria* in the PI group were higher than those in the PT group.

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Buccal mucosa Supragingiva Subgingiva

Relative abundance (%)

100 75 50 25 0

PH PT PI PH PT PI PT PI

A

Archaea
Euryarchaeota
Thaumarchaeota
Bacteria
Acidobacteria
Actinobacteria
Bacteroidetes
Cyanobacteria
Deferribacteres
Deinococcus.Thermus
Firmicutes
Fusobacteria
Gemmatimonadetes
Planctomycetes
Proteobacteria
Spirochaetes
Synergistetes
Tenericutes
Verrucomicrobia
Unassigned

Buccal mucosa Supragingiva Subgingiva

Relative abundance (%)

100 75 50 25 0

PH PT PI PH PT PI PT PI

B

Streptococcus
Neisseria
Haemophilus
Fusobacterium
Rothia
Capnocytophaga
Porphyromonas
Lautropia
Treponema
Prevotella
Leptotrichia
Veillonella
Selenomonas
Gemella
Actinomyces
Campylobacter
Corynebacterium
Peptostreptococcus
Fretibacterium
Tannerella
Cardiobacterium
Parvimonas
Aggregatibacter
Filifactor
Lachnoanaerobaculum
Chryseobacterium
Schwartzia
Eikenella
Schaalia
Dialister

Figure 2. Relative abundance of the microbial composition at the (A) phylum level and (B) genus level according to disease status and sampling sites. Graphs show the proportions of mean relative abundances of each phylum or genus, which were calculated from the samples within the respective subjects.
PH: periodontally healthy, PI: peri-implantitis, PT: periodontitis.

At the genus level, 10 genera (*Streptococcus*, *Neisseria*, *Haemophilus*, *Fusobacterium*, *Rothia*, *Capnocytophaga*, *Porphyromonas*, *Lautropia*, *Treponema*, and *Prevotella*) comprised more than 50% of the total sequences in each group (**Figure 2B**). Compared with those of the PH and PT subjects, the bacterial communities in the PI subjects had higher levels of *Haemophilus*, *Fusobacterium*, *Porphyromonas*, and *Leptotrichia* in the supragingival samples; and *Fusobacterium*, *Porphyromonas*, *Leptotrichia*, *Veillonella*, *Corynebacterium*, and *Peptostreptococcus* in the subgingival samples.

Comparison of significantly different relative abundances

Several species in the diseased groups presented significantly different relative abundances compared to those in the PH group in the buccal (PT: 21 species, PI: 51 species) (**Figure 3A and B**) and supragingival samples (PT: 29 species, PI: 68 species) (**Figure 3C and D**). In the buccal samples, the relative abundances of *Eubacterium* spp., *Treponema* spp., *Porphyromonas gingivalis*, *Filifactor alocis*, *Mogibacterium timidum*, *Parvimonas micra*, *Fretibacterium fastidiosum*, and *Mycoplasma faucium* were high in both the PT and PI groups. In the supragingival samples, the relative abundances of *Eubacterium nodatum*, *Eubacterium saphenum*, *Treponema* spp., *Prevotella intermedia*, *P. gingivalis*, *F. alocis*, *M. timidum*, *F. fastidiosum*, and *M. faucium* were high in both diseased groups.

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Figure 3. Significantly different relative abundances of microbiota according to (A) the disease status, (B) buccal mucosa, (C) supragingival plaque, and (D) supragingival plaque. These were identified by LDA coupled with effect size measurements. Only taxa that met the LDA significance threshold of 2.0 are shown.
LDA: linear discriminant analysis, PH: periodontally healthy, PI: peri-implantitis, PT: periodontitis.

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When comparing the diseased groups, 57, 44, and 67 species in the buccal, supragingival, and subgingival samples, respectively, exhibited significantly higher relative abundances in the PI group than in the PT group (**Figure 4A-C**). These include *P. gingivalis*, *E. nodatum*, *Pseudoramibacter alactolyticus*, *Solobacterium moorei*, and *Porphyromonas endodontalis*. In contrast, considerably fewer species (7, 11, and 13 in the buccal, supragingival, and subgingival samples, respectively) presented significantly higher relative abundances in the PT group than in the PI group, including *Capnocytophaga* spp., *Campylobacter* spp., and *Porphyromonas catoniae.*

Relative abundances of significant bacterial species

Well-known periodontal pathogens, such as *P. gingivalis*, *Prevotella* spp., *Treponema* spp., *F. alocis*, and *F. fastidiosum* were prevalent in both the PT and PI groups, and most showed high relative abundances (**Figure 5**). In contrast, several bacteria previously unrecognized in periodontal tissue, such as *Anaerotignum lactatifermentans*, *Bacteroides vulgatus*, *Faecalibacterium prausnitzii*,

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Supragingiva

Porphyromonas pasteri
Prevotella pallens
Prevotella intermedia
Streptococcus anginosus
Treponema bryantii
Streptobacillus
Selenomonas infelix
Mogibacterium timidum
Blautia
Eubacterium saphenum
Mycoplasma faucium
Fretibacterium fastidiosum
Stomatobaculum longum
Campylobacter rectus
Eubacterium nodatum
Bulleidia extructa
Treponema amylovorum
Treponema putidum
Neisseria shayeganii
Atopobium rimae
Porphyromonas gingivalis
Capnocytophaga leadbetteri
Peptoanaerobacter stomatis
Dialister pneumosintes
Schaalia cardiffensis
Filifactor alocis
Leptotrichia
Carnobacteriaceae
Treponema medium
Peptostreptococcus stomatis
Capnocytophaga sputigena

State
PH
PT

−2 0 2 4
LDA score
C

Supragingiva

Olsenella profusa
Neisseria meningitidis
Treponema pectinovorum
Faecalibacterium prausnitzii
Anaerotignum lactatifermentans
Treponema parvum
Desulfovibrio simplex
Prevotella buccae
Eggerthia catenaformis
Parasutterella excrementihominis
Prevotella nanceiensis
Prevotella denticola
Oribacterium asaccharolyticum
Prevotella multiformis
Schaalia meyeri
Bacteroides vulgatus
Bifidobacterium dentium
Veillonella
Schaalia cardiffensis
Dialister pneumosintes
Alloprevotella tannerae
Treponema bryantii
Prevotella baroniae
Eubacterium
Prevotella dentalis
Peptoanaerobacter stomatis
Streptobacillus
Fusobacterium canifelinum
Eubacterium infirmum
Rothia
Tindallia magadiensis
Anaeroglobus geminatus
Peptococcus niger
Streptococcus anginosus
Treponema socranskii
Streptococcus cristatus
Slackia exigua
Streptococcus alactolyticus
Olsenella uli
Selenomonas infelix
Bulleidia extructa
Stomatobaculum longum
Eubaterium brachy
Prevotella intermedia
Treponema amylovorum
Phocaeicola abscessus
Blautia
Pseudoramibacter alactolyticus
Mycoplasma faucium
Dialister invisus
Catonella morbi
Selenomonas sputigena
Mogibacterium timidum
Atopobium rimae
Treponema maltophilum
Streptococcus parasanguinis
Eubactrium saphenum
Leptotrichia goodfellowii
Prevotella oris
Tannerella forsythia
Treponema medium
Treponema putidum
Treponema denticola
Filifactor alocis
Fretibacterium fastidiosum
Parvimonas micra
Peptostreptococcus stomatis
Eubacterium nodatum
Porphyromonas gingivalis
Porphyromonas endodontalis

State
PH
PI

−2 0 2 4
LDA score
D

Figure 3. (Continued) Significantly different relative abundances of microbiota according to (A) the disease status, (B) buccal mucosa, (C) supragingival plaque, and (D) supragingival plaque. These were identified by LDA coupled with effect size measurements. Only taxa that met the LDA significance threshold of 2.0 are shown. LDA: linear discriminant analysis, PH: periodontally healthy, PI: peri-implantitis, PT: periodontitis.

Olsenella uli, *Parasutterella excrementihominis*, *Prevotella buccae*, *P. alactolyticus*, and *Slackia exigua*, were considerably more significantly abundant in the PI group than in the PH and PT groups.

Bacterial correlation networks

The bacterial networks of the supragingival and subgingival samples established more complex and larger clusters than those of the buccal samples. In both the supragingival and subgingival samples, a cluster of bacterial species in the diseased groups (PI and PT) presented cooperative interactions. In the supragingival samples, dense connections were noted between *Treponema* spp., *Eubacterium* spp., *Tannerella forsythia*, *P. gingivalis*, *F. fastidiosum*, and *F. alocis* in both the PT and PI subjects (**Figure 6B**). In the subgingival samples, *Treponema* spp., *T. forsythia*, *M. timidum*, *M. faucium*, *P. endodontalis*, and *F. alocis* formed a dense network (**Figure 6C**). In the buccal samples, the nodes of *E. nodatum* and *F. alocis* were highly connected with other bacteria in the network (**Figure 6A**).

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Figure 4. Significantly different relative abundances of microbiota between subjects with PT and subjects with PI in (A) buccal mucosa, (B) supragingival plaque, and (C) subgingival plaque. The LDA effect size was used to identify taxa that had an abundance significantly different between groups. Only taxa that met the LDA significance threshold of 2.0 are shown.
LDA: linear discriminant analysis, PT: periodontitis, PI: peri-implantitis.

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DISCUSSION

In the shift from symbiosis to dysbiosis, keystone pathogens interfere with host immune reactions and contribute to homeostatic destruction. Moreover, in dysbiosis, pathobionts show increased relative abundance and cause destructive inflammation and bone loss [25]. In implants, this dysbiosis could cause PI and, if untreated, result in destruction of osseointegration, leading to implant loss [26]. Because the microbiome is related to oral disease, the present study analyzed the microbiome in relation to PI. In this study, the clinical periodontal parameters were similar in the PT and PI subjects. However, many more bacterial species showed significantly higher relative abundances in the PI group than in the PT group in both the supragingival and subgingival samples. Given these results, it could be inferred that, compared to PT, PI is a more complex inflammatory response that involves more bacteria.

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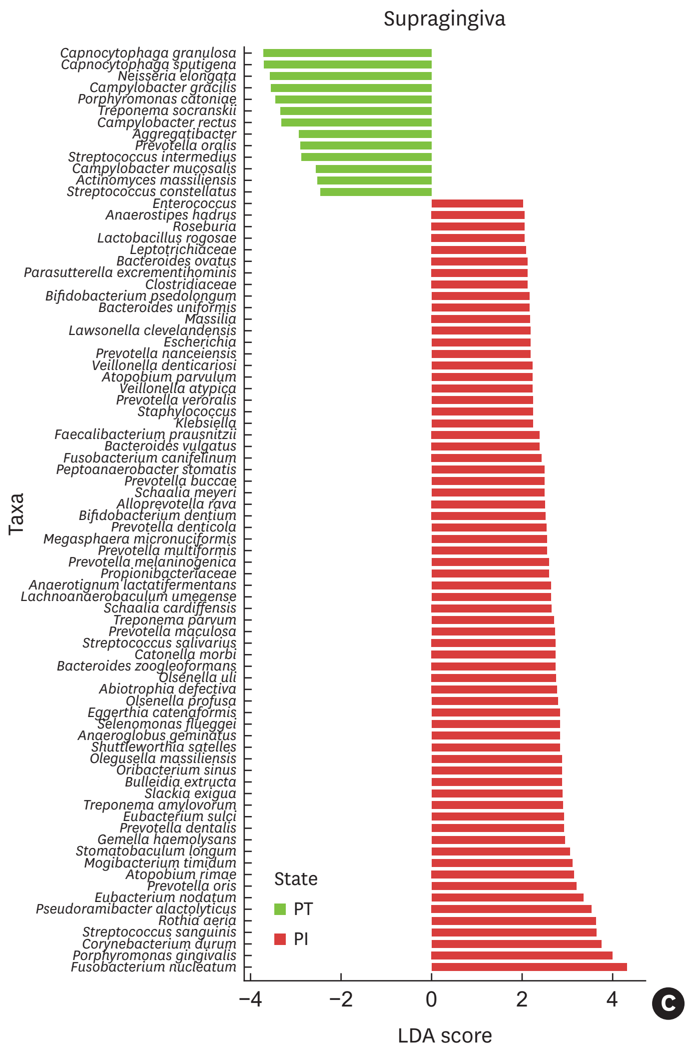

Figure 4. (Continued) Significantly different relative abundances of microbiota between subjects with PT and subjects with PI in (A) buccal mucosa, (B) supragingival plaque, and (C) subgingival plaque. The LDA effect size was used to identify taxa that had an abundance significantly different between groups. Only taxa that met the LDA significance threshold of 2.0 are shown.
LDA: linear discriminant analysis, PT: periodontitis, PI: peri-implantitis.

Both the PT and PI groups presented higher alpha diversity of the microbiota than was observed in the PH group. Specifically, the PI group showed more enrichment of the microbiota than the PT group, which was consistent with the results of previous studies [27,28]. Since inflammation damages the periodontal tissue, thereby supplying an important source of nutrients, more diverse and abundant microbiota could be observed in an inflammatory state than in a healthy state [29]. In this regard, the microbiota may have been more diverse in the PI group than in the PT group because the peri-implant invasion was more vulnerable to the development of inflammation and exhibited a more aggressive response than the periodontal tissue [9]. A previous study also reported that severe inflammation was more likely to occur in peri-implant tissue than in periodontal tissue, leading to differences in the core microbiota between PI and PT [28]. The beta diversity analysis showed significant separation of the microbial clusters of the 3 groups ($P<0.05$) in the buccal samples. Therefore, as microbiome analysis from buccal samples is easy and noninvasive, it could be useful for screening these diseases [14,30]. In the subgingival

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Figure 5. The relative abundances of significant bacterial species. Graphs show the proportion of mean relative abundance for well-known periodontal pathogens and newly identified bacteria within PH, PT, and PI subjects in buccal mucosa, supragingival plaque, and subgingival plaque.
PH: periodontally healthy, PI: peri-implantitis, PT: periodontitis.
[a)]Above significant linear discriminant analysis threshold of 2.0.

Figure 6. Bacterial correlation networks among PH, PT, and PI subjects in (A) buccal mucosa, (B) supragingival plaque, and (C) subgingival plaque. To visualize interactions and characterize the microbial community, the Sparse Correlations for Compositional Data (SparCC) method and Cytoscape were used. The nodes denote the species and the connections represent the existence of correlations.
PH: periodontally healthy, PI: peri-implantitis, PT: periodontitis.

samples, although there was no significant difference, the microbial clusters of PT and PI showed distinct tendencies. A distinctive separation between the PT and PI microbiota was also observed in subgingival samples in a previous study [31].

The relative abundances of specific phyla and genera were higher in the diseased groups than those in the PH group. As the relative abundances of *Firmicutes*, *Spirochaetes*, and *Synergistetes* were higher in both the PT and PI groups than in the PH group, these phyla could be considered to be highly associated with the inflammatory response. When comparing only the diseased groups, some phyla and genera were more abundant in the PI subjects than in the PT subjects. Since PI is a more severe inflammatory response than PT [9] and the subgingival pocket is directly related to the disease condition, the higher abundances of *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, and *Fusobacteria* in the PI group than in the PT group in the subgingival samples of this study may reflect the aggressive progression of PI.

This study identified microbiota with significantly higher relative abundances in both the PT and PI groups than in the PH group. In the buccal and supragingival samples, these bacteria included *M. timidum* and *M. faucium*, as well as several well-known periodontal pathogens (including *P. gingivalis*, *F. alocis*, *F. fastidiosum Treponema* spp., *Eubacterium* spp., *P. intermedia*, and *P. micra*). A previous study reported that *Porphyromonas* spp. and *Prevotella* spp. were more abundant in diseased implants than in healthy implants [32]. *Eubacterium* spp., *T. denicola*, and *P. intermedia* were also reported to be more prevalent in diseased implants [33]. Since the relative abundances of these bacteria are significantly higher in inflammatory conditions, they can be considered as keystone pathogens contributing to the progression of PT or PI, or pathobionts accelerating disease progression by adapting well to the altered environment caused by the disease.

Several bacterial species had significantly different relative abundance levels between the PT and PI subjects. The abundance of *P. gingivalis*, *E. nodatum*, *P. alactolyticus*, *S. moorei*, and *P. endodontalis* was higher in the PI subjects. Most of these bacteria have not been identified in periodontal tissue by conventional methods, but recent studies using NGS also reported the existence of *P. endodontalis*, *F. alocis*, *F. fastidiosum*, *P. alactolyticus*, *S. moorei*, and *Peptostreptococcus*

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stomatis in samples from patients with PI [31]. *P. endodontalis* is considered to be abundant in infected root canals [34], capable of inducing osteoclastogenesis [35], and a core bacterium of PT [36]. A previous study also found *P. stomatis* and *S. moorei* in samples from patients with PI, but their properties are unknown [31]. In particular, the genus *Peptostreptococcus* is known to cause abscesses and necrotic soft tissue infection [37].

Additionally, this study found, at low levels of relative abundance, the presence of bacterial species that have rarely been mentioned in other studies. Compared to those in the PH and PT groups, these bacteria had considerably higher proportions in the PI group, which presented significant relative abundances at all sites. These bacteria included *A. lactatifermentans*, *B. vulgatus*, *O. uli*, *P. excrementihominis*, *P. buccae*, and *S. exigua*. However, there is little information, to our knowledge, on the function of these bacteria in periodontal tissue. *S. exigua* has been reported to be prevalent in periapical infections and has been identified in deep PI pockets [38]. *O. uli* has been found to be abundant in infected root canals [34], but its role in PI is unknown. Moreover, *F. prausnitzii* and *P. alactolyticus* also presented similar tendencies to these bacteria. *P. alactolyticus* is known to be common within canals with irreversible pulpitis [39]. In addition, it has recently been found in diseased implants [40]. Taken together, although most of these bacteria had a substantially low relative abundance and little is known about their role in PI, they might play an important role in the dysbiosis of PI.

In the bacterial community interaction network, some microbiota interacted with other bacteria in both diseased groups. In the network, well-known periodontal pathogens, including the red complex, *Treponema* spp., *Prevotella* spp., *E. nodatum*, *F. alocis*, and *F. fastidiosum* were also found in this study. *T. maltophilum*, *M. timidum*, and *P. endodontalis* were found in this study, but few have been reported in periodontal tissues in the past. Most of these bacteria exhibited dense connectivity with other species in both PT and PI subjects. Moreover, more complex and larger clusters were established in both the supragingival and subgingival samples than in the buccal samples. Therefore, it could be assumed that some of these microbes, as common strains associated with both inflammatory diseases, function like pathobionts.

The mean ages in each subject group (PH, PT, and PI) were 26.9, 50.7, and 55.8 years, respectively. One of the limitations of the present study was that the mean age of PH differed considerably from that of the other groups. Furthermore, in this study, the PH subjects were employed as a control group for the diseased groups. A more systematic comparison would have been possible if subjects with healthy implants were additionally recruited to the control group. Since PT and PI are diseases involving multifactorial risk factors, it is necessary to conduct sampling from the same subjects in order to control for subject-dependent factors in these groups more clearly. Therefore, future research will need to address these limitations.

In conclusion, compared with the PH and PT groups, the composition of the microbiota showed considerable differences in the PI group, and this result could be associated with the aggressive and complicated nature of PI. The microbial profile specific to PI, as identified by NGS, could provide significant evidence relevant for the treatment of this disease. Further research into the role of unique bacteria found in this study in regard to PI will be helpful for establishing an effective and optimal treatment protocol for this disease.

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