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Prevalence and abundance of 9 periodontal pathogens in the saliva of periodontally healthy adults and patients undergoing supportive periodontal therapy

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ABSTRACT

Purpose: This study aimed to examine the prevalence and abundance of 9 representative periodontal pathogens in the saliva samples of periodontally healthy subjects (PH) and patients with periodontitis who underwent supportive periodontal therapy (SPT). The age-specific distribution of these pathogens in periodontally healthy individuals was also analyzed.
Methods: One hundred subjects (aged >35 years) were recruited (50 each in the PH and SPT groups) between August 2016 and April 2019. The prevalence and abundance of periodontal pathogens in the PH group were compared with those in periodontally healthy young subjects (94 subjects; aged <35 years), who were included in our previous study. DNA copy numbers of *Aggregatibacter actinomycetemcomitans* (*Aa*), *Porphyromonas gingivalis* (*Pg*), *Tannerella forsythia* (*Tf*), *Treponema denticola* (*Td*), *Prevotella intermedia (Pi)*, *Fusobacterium nucleatum* (*Fn*), *Campylobacter rectus* (*Cr*), *Peptostreptococcus anaerobius (Pa)*, and *Eikenella corrodens* (*Ec*) were analyzed using real-time polymerase chain reaction.
Results: The detection frequencies of all pathogens, except *Aa*, were high in the PH and SPT groups. The ranking order of pathogen DNA copy numbers was similar in both groups. In both groups, *Fn* had the highest abundance, *Aa* had the lowest abundance. Additionally, *Td* was significantly more abundant in men than in women in both groups ($P<0.05$). Compared with the PH group, the SPT group exhibited significantly lower total bacteria and *Fn* abundance and higher *Pg* abundance ($P<0.05$). The age-specific pathogen distribution analysis revealed a significantly low *Aa* abundance and high *Tf* and *Cr* abundance in the PH group.
Conclusions: The clinical parameters and microbial profiles were similar between the SPT and PH groups. However, patients with periodontitis require supportive care to prevent recurrence. As the abundance of some bacteria varied with age, future studies must elucidate the correlation between age-related physiological changes and periodontal bacterial composition.

Keywords: Adult; Aging; Bacterial load; Chronic periodontitis; Maintenance; Saliva

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Author Contributions

Conceptualization: Ju-Youn Lee, Woo-Ri Jung; Data curation: Hyun-Joo Kim; Formal analysis: Woo-Ri Jung; Funding acquisition: Ju-Youn Lee; Investigation: Ju-Youn Lee, Woo-Ri Jung, Hyun-Joo Kim; Methodology: Ju-Youn Lee, Ji-Young Joo; Project administration: Ju-Youn Lee, Hyun-Joo Kim; Writing - original draft: Woo-Ri Jung, Hyun-Joo Kim; Writing - review and editing: Ju-Youn Lee, Ji-Young Joo, Hyun-Joo Kim.

Conflict of Interest

No potential conflict of interest relevant to this article is reported.

INTRODUCTION

Periodontal disease was previously considered an infectious disease caused by periodontal pathogens. Since the 2000s, periodontal disease has been reported to have multifactorial etiologies involving periodontal pathogens and various other factors, such as the host immune system, genetics, and environmental factors [1]. In periodontally healthy individuals, a state of equilibrium exists between the pathogen population and host immune response. The disruption of this equilibrium changes the oral microflora composition in the biofilm, which results in periodontal disease and periodontal tissue destruction [2,3]. Thus, the transition from a healthy periodontal state to a diseased state is caused by both periodontal pathogens and subgingival dysbiosis [1].

Several studies have analyzed the oral bacterial composition of patients with periodontitis [4-12]. The most common pathogen causing periodontitis is the red complex, comprising *Porphyromonas gingivalis* (*Pg*), *Tannerella forsythia* (*Tf*), and *Treponema denticola* (*Td*). Periodontitis is also caused by the orange (*Prevotella intermedia* [*Pi*], *Fusobacterium nucleatum* [*Fn*], and *Micromonas micros*) and green (*Eikenella corrodens* [*Ec*] and *Aggregatibacter actinomycetemcomitans* [*Aa*]) complexes [12,13]. These bacteria are also detected in the oral cavity of periodontally healthy individuals. Therefore, an investigation of the oral microflora composition in periodontally healthy individuals will help diagnose periodontitis and predict disease risk [1,6,14-16].

The prevalence and severity of periodontitis increase with age, which may be associated with immunosenescence or inflamm-aging [17,18]. Previous studies have analyzed age-related changes in the composition and distribution of periodontal microbiota in periodontally healthy subjects (PH). Percival et al. [19] examined the age-dependent changes in the oral microbiota composition in the oral biofilm and saliva samples of periodontally healthy adults and reported that the total counts of viable microbes did not vary among different age groups. Recently, Preza et al. [20] identified various bacterial species in the oral cavities of periodontally healthy elderly individuals. Compared with the young and middle-age groups, the elderly group exhibited markedly different bacterial profiles. Further studies are needed to examine age-related changes in the composition and distribution of periodontal pathogens [17].

Periodontal treatment reduces the number of periodontal pathogens and improves clinical parameters. Additionally, supportive periodontal therapy (SPT) maintains the stability of periodontal treatment outcomes [21,22]. One study reported that initial periodontal therapy comprising scaling and root planing was effective in reducing the probing pocket depth, improving the attachment level, and decreasing the number of *Pg*, *Pi*, and *Aa* at the treatment sites [23]. Therefore, the composition of oral microbiota in patients undergoing SPT after periodontal treatment may be different from that of patients with periodontitis. Studies on the differences in the composition of periodontal pathogens between PH and patients undergoing SPT following periodontal treatment are limited.

Feres et al. [18] classified patients based on age as follows: young adults (<35 years old), adults (≥35 and <65 years old), and older adults (≥65 years old). Previously, we analyzed the composition of periodontal pathogens in the saliva samples of periodontally healthy Korean young adults [24]. The present study aimed to examine the detection frequencies and abundance of 9 representative periodontal pathogens in the saliva samples of periodontally healthy Korean adults aged over 35 years using quantitative real-time

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polymerase chain reaction (qRT-PCR). The composition of periodontal pathogens was also comparatively analyzed between periodontally healthy individuals with no history of periodontitis (PH group; aged >35 years) and patients undergoing SPT (SPT group; aged >35 years) to determine the correlation of clinical restoration of periodontal health with the oral microbiota composition and to evaluate the possibility of predicting periodontitis recurrence. Furthermore, the distribution of pathogens in the PH group was compared with that in periodontally healthy young adults (PHY group; aged <35 years), who were included in our previous study [24], to analyze the age-specific distribution of pathogens.

MATERIALS AND METHODS

Subject population and clinical measurements

In total, 100 subjects who visited the Pusan National University Dental Hospital between August 2016 and December 2019 were enrolled in the present study. Of these 100 subjects, 50 (30 men and 20 women) comprised the PH group and 50 (22 men and 28 women) comprised the SPT group. The clinical and experimental data of the 94 patients in the PHY group from our previous study [24] were used to analyze the distribution of bacteria among different age groups. The exclusion criteria were as follows: individuals with uncontrolled systemic diseases affecting periodontal health; patients who received periodontal treatment in the previous 6 months (except for the SPT group); pregnant or breastfeeding patients; individuals who did not sign the informed consent form; and individuals with fewer than 20 teeth. In the PH group, subjects with a uniform probing depth (PD) of less than 3 mm, no evidence of attachment loss, and no signs of gingival inflammation were selected. The SPT group comprised patients who maintained stable periodontal health after resolving periodontal tissue inflammation through active periodontal treatment. They had received SPT at intervals of 3 to 6 months with good compliance for at least 2 years after active periodontal treatment. The inclusion criteria for the SPT group were having a uniform PD of less than 3 mm and no signs of gingival inflammation. One periodontal specialist recorded the clinical parameters, including PD, clinical attachment level (CAL), gingival recession (GR), plaque index (PI), and gingival index (GI). This study was approved by the Institutional Review Board of Pusan National University Dental Hospital (PNUDH-2016-019). All study participants were informed about the purpose and process of the study along with the potential risks, and all of them signed written informed consent forms prior to participating in the current study.

Human saliva sample collection and bacterial DNA extraction

Saliva samples were collected from each of the 100 subjects after the individuals gargled 12 mL of a solution (E-zen Gargle, JN Pharm, Pyeongtaek, Korea) for 30 seconds without brushing. The collected samples were labeled and stored at 4°C until analysis. To remove the residues and precipitates, the samples in phosphate-buffered saline were centrifuged at 3,900 rpm for 10 minutes. Bacterial DNA was extracted from the samples using a DNA extraction kit (Exgene™ Clinic SV, GeneAll Biotechnology, Seoul, Korea), following the manufacturer's instructions. The quality and quantity of extracted DNA were examined using a spectrophotometer (Nanodrop, Thermo Fisher Scientific, Waltham, MA, USA).

Nine microbial strains

In this study, the following 9 microbial strains were analyzed: *Aa* (KCCM 12227), *Pg* (KCTC 5352), *Tf* (KCTC 5666), *Td* (KCTC 15104), *Pi* (KCTC 5694), *Fn* (KCTC 2640), *Peptostreptococcus anaerobius* (*Pa*; KCTC 5182), *Campylobacter rectus* (*Cr*; KCTC 5636), and *Ec* (KCTC 15198).

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Table 1. Primers and probes used for quantitative polymerase chain reaction analysis

Species	Sequence (5′-3′)	Product size (16S rRNA)
Aggregatibacter actinomycetemcomitans		80 bp
Forward primer	GAACCTTACCTACTCTTGACATCCGAA	
Reverse primer	TGCAGCACCTGTCTCAAAGC	
Probe	FAM-GAACTCAGAGATGGGTTTGTGCCTTAGGG	
Porphyromonas gingivalis		344 bp
Forward primer	TGCAACTTGCCTTACAGAGGG	
Reverse primer	ACTCGTATCGCCCGTTATTC	
Probe	ABY-AGCTGTAAGATAGGCATGCGTCCCATTAGCTA	
Tannerella forsythia		126 bp
Forward primer	GGGTGAGTAACGCGTATGTAACCT	
Reverse primer	CCCATCCGCAACCAATAAA	
Probe	VIC-CCCGCAACAGAGGGATAACCCGG	
Treponema denticola		204 bp
Forward primer	TGGTGAGTAACGCGTGGGTGACCT	
Reverse primer	TTCACCCTCTCAGGCCGGA	
Probe	ABY-CCTGAAGATGGGGATAGCTAGTAGA	
Prevotella intermedia		232 bp
Forward primer	CCACATATGGCATCTGACGTG	
Reverse primer	CACGCTACTTGGCTGGTTCA	
Probe	FAM-ACCAAAGATTCATCGGTGGAGGATGGG	
Fusobacterium nucleatum		163 bp
Forward primer	GGATTTATTGGGCGTAAAGC	
Reverse primer	GGCATTCCTACAAATATCTACGAA	
Probe	FAM-CTCTACACTTGTAGTTCCG	
Campylobacter rectus		121 bp
Forward primer	TTTCGGAGCGTAAACTCCTTTTC	
Reverse primer	TGATTCCGAGTAACGCTTGCA	
Probe	VIC-GGGAAAGAATTATGACGGTA	
Peptostreptococcus anaerobius		181 bp
Forward primer	GGGTGAGTAACGCGTGGGT	
Reverse primer	TACTGATCGTCGCCTTGGTGG	
Probe	VIC-ATGTTATCCATGTGTATAGGGC	
Eikenella corrodens		197 bp
Forward primer	ACGTCCTACGGGAGAAAGCGG	
Reverse primer	CCATTGTCCAAAATTCCCCACTG	
Probe	ABY-CTCGCGTTATTCGAGCGGCCGATA	
Total bacteria		159 bp
Forward primer	TGGAGCATGTGGTTTAATTCGA	
Reverse primer	TGCGGGACTTAACCCAACA	
Probe	JUN-CACGAGCTGACGACA(AG)CCATGCA	

Primers and probes used for qRT-PCR

The primers and probes used for qRT-PCR analysis are shown in Table 1. The variable regions of pathogen 16S rRNA were amplified using species-specific primers and probes. Additionally, the universal bacterial primer pair and probe were used to examine the abundance of total bacteria in the saliva samples. The fluorescent dyes at the 5′ ends of the probe were FAM, VIC, ABY, and JUN. These dyes were used to perform multiplex experiments and detect up to 4 targets in a single reaction.

Standard curves and multiplex RT-PCR

To obtain a standard curve, the 16S rRNA region of each target bacterium was cloned using a PCR cloning vector system (pGEM®-T Easy Vector System, Promega, Madison, WI, USA). The plasmid DNA sequences were examined using Sanger sequencing. The purified plasmids were quantified using the TaqMan® RT-PCR system. The standard curves were prepared using 10-fold serially diluted (from 10^2 to 10^9) plasmid DNA samples. The plasmid standards

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were run in triplicate, and the mean values were used to calculate the copy numbers of bacteria. The qRT-PCR analysis was performed in a 20-μL reaction volume comprising 2 μL of template, 400 nM primers, and 100 nM probe using qRT-PCR kits (2× TaqMan Multiplex Master Mix, Applied Biosystems, Foster City, CA, USA). For analyzing *Tf* and *Pi*, 900 nM primers and 100 nM probe were used. The qRT-PCR conditions for generating the standard curves were as follows: denaturation at 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds, 50°C for 30 seconds, and 72°C for 30 seconds. An uracil-DNA glycosylase incubation step was performed before PCR cycling to prevent carryover contamination. All qRT-PCR analyses were performed using a RT-PCR system (QuantStudio™ 6 Flex Real-Time PCR System, Thermo Fisher Scientific). The combination of primers and probes for the multiplex reaction was determined based on the cycle threshold (Ct) values. The changes in Ct values between singleplex and multiplex reactions (ΔCt) were less than 0.3. Finally, 3 multiplex reactions were performed for each sample: total bacteria, *Aa*, *Cr*, and *Ec* in the first reaction; *Fn*, *Pa*, and *Pg* in the second reaction; and *Pi*, *Tf*, and *Td* in the third reaction.

Statistical analysis

All statistical analyses were performed using SPSS version 24 (IBM Corp., Armonk, NY, USA). The differences in the number of pathogens between different groups were analyzed using analysis of variance, followed by Scheffé *post hoc* analysis. The differences in the DNA copy number of pathogens between the genders were analyzed using the independent *t*-test. The differences were considered significant at $P<0.05$.

RESULTS

Demographic and clinical characteristics

The present study comprised 50 subjects in the PH group (30 men and 20 women) and 50 subjects in the SPT group (22 men and 28 women). The mean age of the PH and SPT groups was 47.90±12.12 and 50.30±5.36 years, respectively. The PD, CAL, GR, PI, and GI values in the PH group were 2.37±0.26 mm, 2.47±0.28 mm, 0.09±0.16 mm, 22.22±16.90, and 0.19±0.23, while those in the SPT group were 2.25±0.44 mm, 2.83±0.56 mm, 0.58±0.47 mm, 15.85±9.24, and 0.10±0.10, respectively (Table 2). The PI and GI values in the PH group were significantly higher than those in the SPT group ($P<0.05$). The CAL and GR values in the PH group were significantly lower than those in the SPT group ($P<0.001$). To analyze the age-specific distribution of pathogens, the clinical data of 94 patients in the PHY group (49 men and 45 women) were included. The PD, CAL, GR, PI, and GI values in the PHY group were 2.41±0.30 mm, 2.43±0.30 mm, 0.02±0.04 mm, 17.25±13.56, and 0.07±0.13, respectively (Table 2). The GI and GR values were significantly higher in the PH group than in the PHY group.

Table 2. Clinical characteristics of PH group (>35 years), patients with periodontitis who underwent SPT group (>35 years), and PHY group (<35 years)

Variables	PH group	SPT group	PHY group
Age (yr)	47.90±12.12	50.30±5.36	28.83±2.34
Gender (%men)	60.0	44.0	52.1
Plaque index	22.22±16.90	15.85±9.24[a)]	17.25±13.56
Gingival index	0.19±0.23	0.10±0.10[a)]	0.07±0.13[c)]
Probing pocket depth (mm)	2.37±0.26	2.25±0.44	2.41±0.30
Clinical attachment level (mm)	2.47±0.28	2.83±0.56[b)]	2.43±0.30
Gingival recession (mm)	0.09±0.16	0.58±0.47[b)]	0.02±0.04[c)]

Values are expressed as the mean±standard deviation or percentage.
PH: periodontally healthy subjects, SPT: supportive periodontal therapy, PHY: periodontally healthy young subjects.
[a)]$P<0.05$; [b)]$P<0.001$: statistical significance of PH versus SPT; [c)]$P<0.05$: statistical significance of PH versus PHY.

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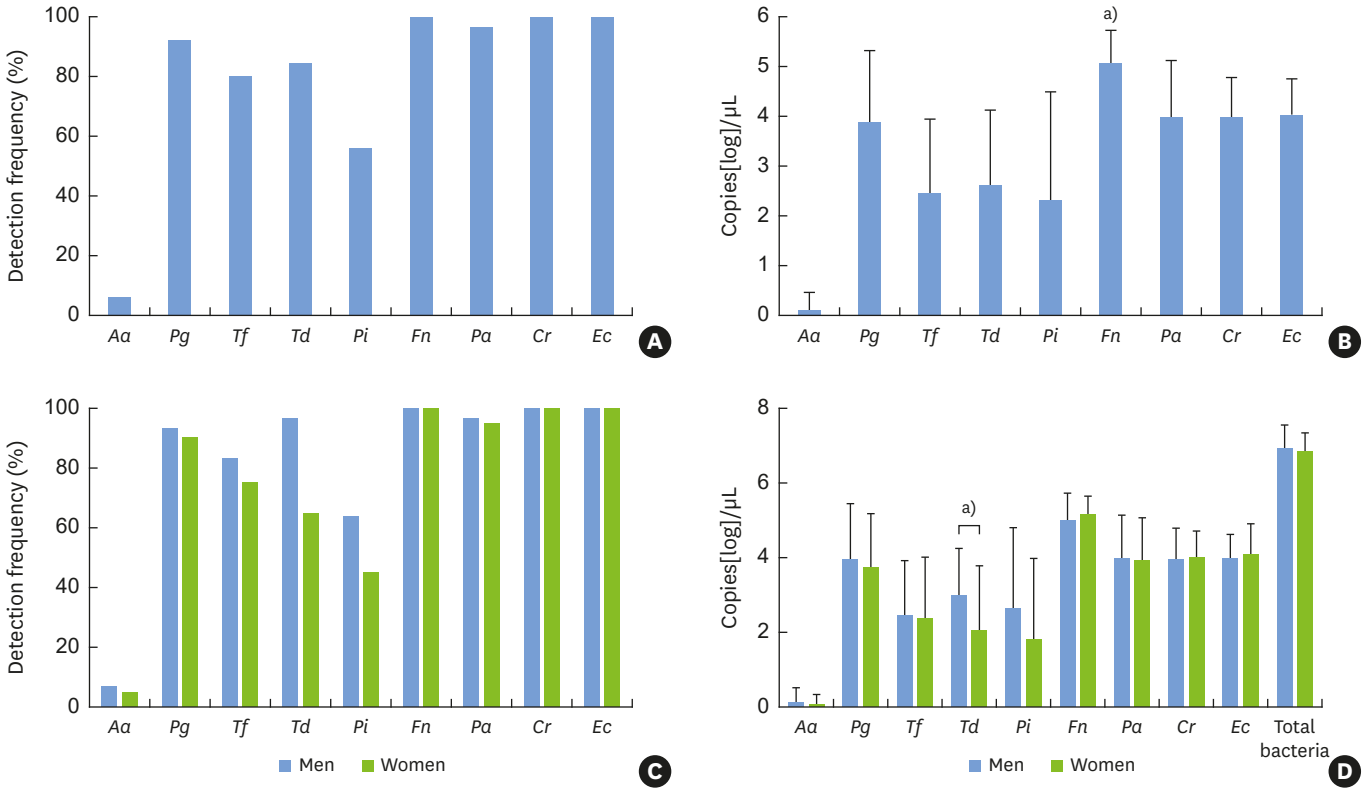

Figure 1. Distribution of 9 periodontal pathogens in PH group (aged >35 years). Detection frequencies (A) and DNA copy numbers (B) of 9 periodontal pathogens in the PH group. (C) Detection frequencies of 9 periodontal pathogens among men and women in the PH group. (D) Comparative analysis of DNA copy numbers of each pathogen and total bacteria among men and women in the PH group. Values are represented as the mean±standard deviation or percentage.
PH: periodontally healthy subjects, *Aa: Aggregatibacter actinomycetemcomitans*, *Cr: Campylobacter rectus*, *Ec: Eikenella corrodens*, *Fn: Fusobacterium nucleatum*, *Pa: Peptostreptococcus anaerobius*, *Pg: Porphyromonas gingivalis*, *Pi: Prevotella intermedia*, *Td: Treponema denticola*, *Tf: Tannerella forsythia*.
[a]$P<0.05$.

Distribution characteristics of the 9 periodontal pathogens in the PH group

The detection frequencies and abundance of the 9 representative periodontal pathogens in the PH group were analyzed using qRT-PCR. The detection rate of each pathogen relative to the number of saliva samples is shown in Figure 1A. *Fn*, *Cr*, and *Ec* were detected in all samples (100%) of the PH group. The detection frequencies of *Pa*, *Pg*, *Td*, and *Tf* in the PH group were 96.0%, 92.0%, 84.0%, and 40%, respectively. However, the detection frequencies of *Pi* and *Aa* were low in the PH group (56.0% and 6.0%, respectively).

The DNA copy numbers of the 9 periodontal pathogens in the saliva samples of the PH group are shown in Figure 1B. The abundance of *Fn* (5.05±0.65) was the highest in the PH group ($P<0.05$), followed by *Ec* (4.02±0.72), *Pa* (3.96±1.15), *Cr* (3.96±1.15), and *Pg* (3.87±1.45), which exhibited similar abundance. Furthermore, the DNA copy numbers of *Td* (2.60±1.53), *Tf* (2.42±1.52), *Pi* (2.30±2.19), and *Aa* (0.09±0.37) were lower than those of the other pathogens.

The detection frequencies and DNA copy numbers of the 9 pathogens among men and women in the PH group are shown in Figure 1C and D. The DNA copy number of *Td* in men (2.97) was significantly higher than that in women (2.05).

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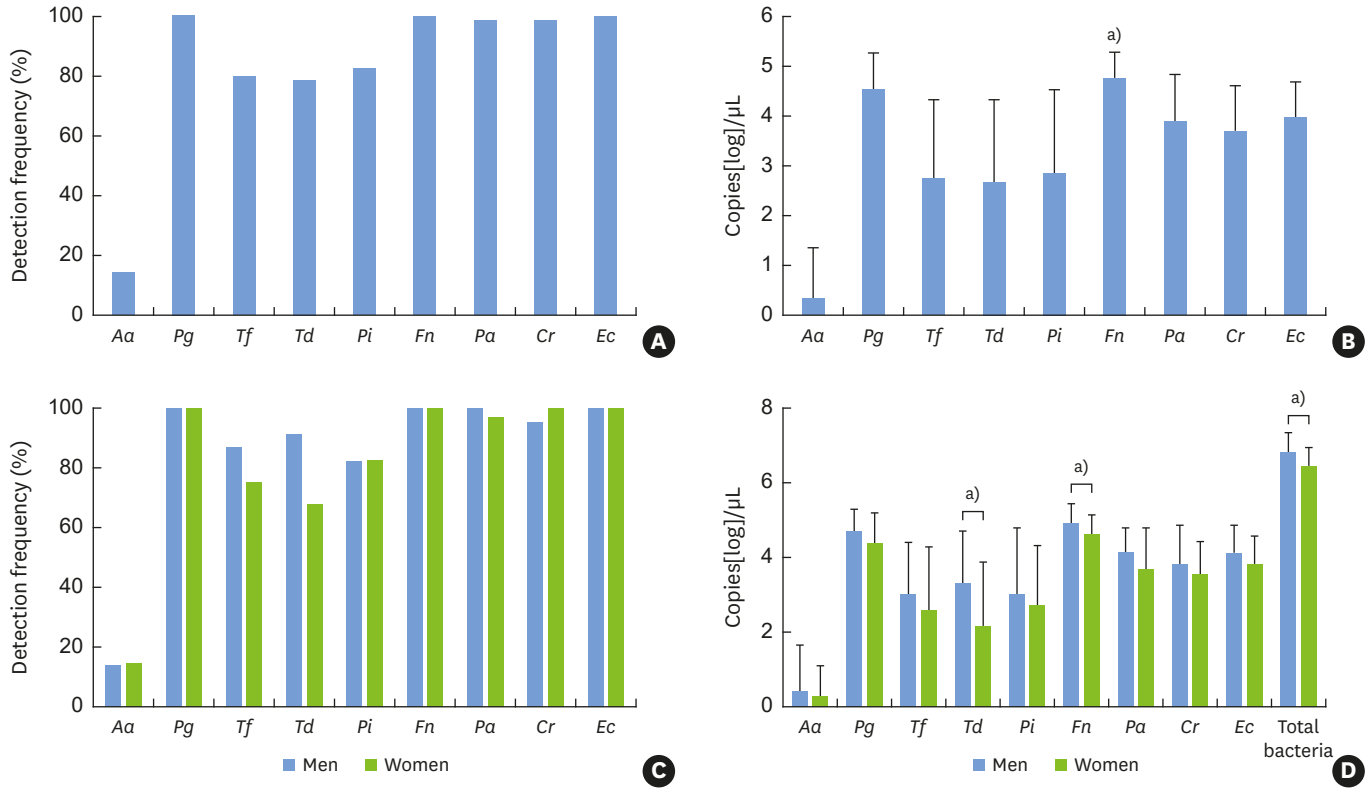

Figure 2. Distribution of 9 periodontal pathogens in patients with periodontitis who underwent SPT group (aged >35 years). Detection frequencies (A) and DNA copy numbers (B) of 9 periodontal pathogens in the SPT group. (C) Detection frequencies of 9 periodontal pathogens among men and women in the SPT group. (D) Comparative analysis of DNA copy numbers of each pathogen and total bacteria among men and women in the SPT group. Values are represented as the mean±standard deviation or percentage.
SPT: supportive periodontal therapy, *Aa: Aggregatibacter actinomycetemcomitans*, *Cr: Campylobacter rectus*, *Ec: Eikenella corrodens*, *Fn: Fusobacterium nucleatum*, *Pa: Peptostreptococcus anaerobius*, *Pg: Porphyromonas gingivalis*, *Pi: Prevotella intermedia*, *Td: Treponema denticola*, *Tf: Tannerella forsythia*.
[a]$P<0.05$.

Distribution characteristics of the 9 periodontal pathogens in the SPT group

The detection frequencies of the 9 periodontal pathogens in the saliva samples of the SPT group are shown in Figure 2A. *Pg*, *Fn*, and *Ec* were detected in all saliva samples of the SPT group. The detection frequencies of *Pa*, *Cr*, *Pi*, *Tf*, *Td*, and *Aa* in the saliva samples of the SPT group were 98%, 98%, 82%, 80%, 78%, and 14%, respectively.

The DNA copy numbers of the 9 periodontal pathogens in the saliva samples of the SPT group are shown in Figure 2B. The most abundant periodontal pathogen in the SPT group was *Fn*. The abundance of *Fn* was significantly higher than that of *Pi*, *Td*, *Aa*, and *Tf* ($P<0.05$). Meanwhile, the abundance of *Pg*, *Pa*, and *Ec* was higher than that of *Pi*, *Td*, and *Tf*. The DNA copy number of *Aa* was the lowest in the SPT group. The detection frequencies and DNA copy numbers of the 9 pathogens among men and women in the SPT group are shown in Figure 2C and D. In the SPT group, the abundance of *Td*, *Fn*, and total bacteria in men was significantly higher than that in women ($P<0.05$).

Comparative analysis of the 9 periodontal pathogens between the PH and SPT groups

The comparative analysis of DNA copy numbers of the 9 periodontal pathogens between the PH and SPT groups is shown in Figure 3. The ranking order of DNA copy numbers of the 9

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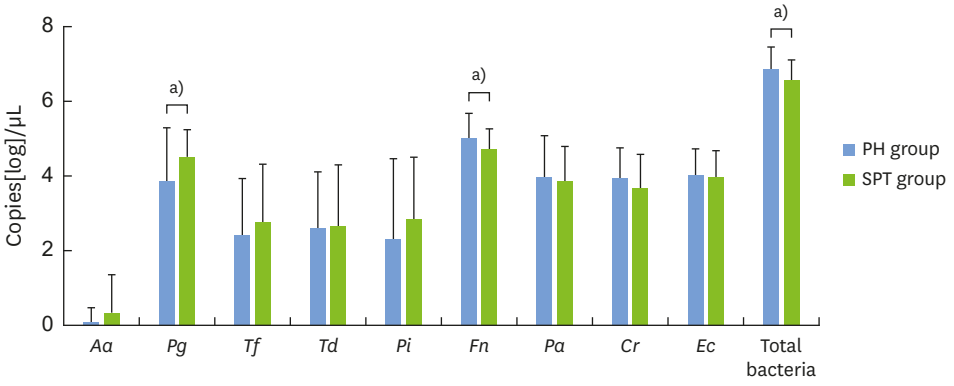

Figure 3. Comparative analysis of DNA copy numbers of 9 periodontal pathogens and total bacteria in PH group (aged >35 years) and patients with periodontitis who underwent SPT group (aged >35 years). Values are represented as mean±standard deviation or percentage.
PH: periodontally healthy subjects, SPT: supportive periodontal therapy, *Aa: Aggregatibacter actinomycetemcomitans*, *Cr: Campylobacter rectus*, *Ec: Eikenella corrodens*, *Fn: Fusobacterium nucleatum*, *Pa: Peptostreptococcus anaerobius*, *Pg: Porphyromonas gingivalis*, *Pi: Prevotella intermedia*, *Td: Treponema denticola*, *Tf: Tannerella forsythia*.
[a)]$P<0.05$.

periodontal pathogens was similar between the PH and SPT groups. However, the abundance of *Pg* and *Fn* significantly varied between the 2 groups. Compared with the PH group, the SPT group exhibited a higher abundance of *Pg* and a lower abundance of *Fn*. The abundance of total bacteria was significantly higher in the PH group than in the SPT group ($P<0.05$).

Comparative analysis of the 9 periodontal pathogens based on age

The comparative analysis of the DNA copy number of the 9 periodontal pathogens among the PH and PHY groups is shown in Figure 4. The DNA copy number data for the PHY group reported in our previous study were used [24]. The abundance of *Tf* and *Cr* was higher in the PH group than in the PHY group. In contrast, the abundance of *Aa* and total bacteria was higher in the PH group than in the PHY group ($P<0.05$). The abundance of *Pg*, *Td*, *Pi*, *Fn*, *Pa*, and *Cr* was not significantly different between the PHY and PH groups.

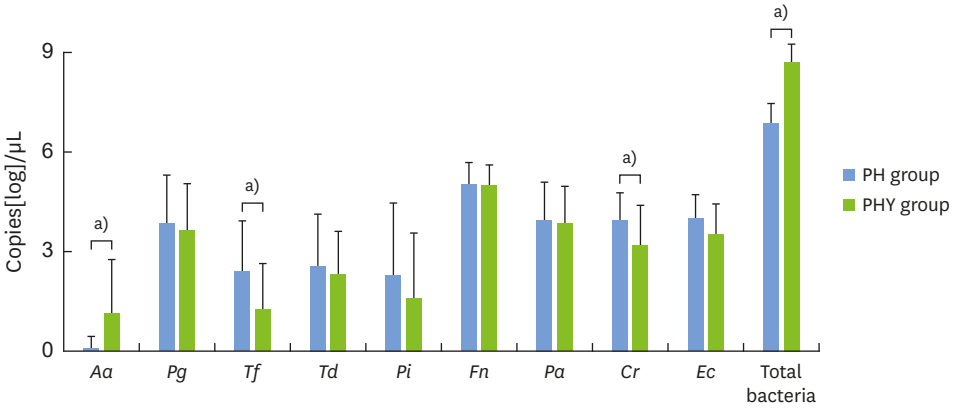

Figure 4. Comparative analysis of the DNA copy number of 9 periodontal pathogens and total bacteria in PH group (aged >35 years) and PHY group (aged <35 years). Values are presented as the mean±standard deviation or percentage.
PH: periodontally healthy subjects, PHY: periodontally healthy young subjects, *Aa: Aggregatibacter actinomycetemcomitans*, *Cr: Campylobacter rectus*, *Ec: Eikenella corrodens*, *Fn: Fusobacterium nucleatum*, *Pa: Peptostreptococcus anaerobius*, *Pg: Porphyromonas gingivalis*, *Pi: Prevotella intermedia*, *Td: Treponema denticola*, *Tf: Tannerella forsythia*.
[a)]$P<0.05$.

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DISCUSSION

Periodontitis is a multifactorial chronic inflammatory disease, but the onset of disease is necessarily linked to pathological changes in the bacterial community. In the present study, the salivary levels of 9 periodontal pathogens associated with periodontitis were analyzed in the PH and SPT groups using qRT-PCR. Periodontal pathogens are predominantly present in subgingival plaque, gingival crevicular fluid, and saliva. Compared to samples obtained from other sites, saliva samples have been attracting attention due to their advantages of being non-invasive, costing less, and having low sensitivity to technique. Saliva samples have been used in many studies as an alternative to subgingival plaque samples for the detection of periodontal pathogens [7]. Several studies have reported that the presence of bacteria in saliva, as well as the number of salivary bacteria, were associated with clinical variables in periodontitis [8,25].

The periodontal health status of the PH and SPT groups was compared based on the clinical parameters. The CAL and GR values in the SPT group were significantly higher than those in the PH group. However, the PD values were similar between the SPT and PH groups. This suggests that periodontal therapy decreased the PD, which resulted in GR. The PI and GI values in the SPT group were significantly lower than those in the PH group. This indicated that the SPT group exhibited good oral hygiene and decreased gingival inflammation. Thus, following active periodontal treatment, SPT restores periodontal tissue health without inflammation at the levels observed in the PH group.

Among the 9 representative periodontal pathogens, the detection frequencies of 8 pathogens (except *Aa*) were more than 50% in the PH group. This is consistent with the results of previous studies, which reported the presence of periodontal pathogens in periodontally healthy individuals [3,26]. The detection frequencies of periodontal pathogens, except *Aa*, in the SPT group were more than 70%. Of the 9 periodontal pathogens, the detection frequency and abundance of *Fn* were significantly higher than those of other bacteria in the PH and SPT groups. *Fn* is a Gram-negative anaerobic periodontal pathogen associated with various human diseases. The abundance of *Fn*, which is also detected in the saliva, increases with the severity of periodontitis, progression of inflammation, and increased PD. Additionally, *Fn* is involved in dental plaque formation and is a link between early and late colonizers in the dental biofilm [27,28]. Previous studies have reported that *Fn* contributes to the aggregation of other periodontal pathogens and that the increased abundance of *Fn* dysregulates immune function by inducing apoptosis in immune cells. Furthermore, *Fn* may play an important role in the progression of early periodontitis [29]. *Aa*, a periodontal pathogen, is frequently detected in adolescents and adults with aggressive forms of periodontitis. In the current study, the detection frequency and abundance of *Aa* were the lowest among the 9 periodontal pathogens in the PH and SPT groups. Similarly, *Aa* was detected at a low frequency (≤13.3%) in a study analyzing subgingival bacteria in Korean patients with chronic periodontitis and periodontally healthy individuals [6]. The microbial analysis of subgingival plaque samples from Korean and German patients with chronic periodontitis revealed that the abundance of *Aa* in German patients (47.7%) was significantly higher than that in Korean patients (26.7%) [30]. This indicates that the profile of oral microorganisms may differ based on race and ethnicity. In the Korean population, the abundance of *Aa* may be poorly correlated with periodontitis.

The detection frequencies and abundance of all 9 pathogens in men were higher than those in women. In particular, the abundance of *Td* in men was significantly higher than that in women in both the PH and SPT groups. In the SPT group, the abundance of *Fn* and total

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bacteria in men was significantly higher than that in women. Previous epidemiological studies have reported that men have a higher risk of developing destructive periodontitis than women owing to their oral hygiene habits, smoking, and environmental factors [31]. Previously, we reported that the abundance of pathogens was not significantly different between the genders; however, the abundance of total bacteria was significantly higher in men than in women [24].

Several studies have reported the characteristics of periodontal pathogens in patients who have undergone SPT. Haffajee et al. [32] compared the subgingival microbiota composition among periodontally healthy individuals, patients undergoing SPT after periodontal treatment, and patients with periodontitis [33]. They reported that subgingival microbiota composition in patients undergoing SPT was similar to that in periodontally healthy individuals, but was different from that in patients with periodontitis. Another study reported that the average oral microbial composition of patients with periodontitis who underwent SPT did not recover to the levels observed in periodontally healthy individuals. This indicated that SPT did not decrease the abundance of all subgingival pathogenic species to the levels observed in periodontally healthy individuals [33]. In the present study, the ranking order of the DNA copy numbers of the 9 periodontal pathogens was similar between the PH and SPT groups. Among these pathogens, *Fn* was the most abundant, while *Aa* was the least abundant in the PH and SPT groups. The 2 groups exhibited similar overall abundance of the 9 periodontal pathogens. This indicates that the application of SPT after active periodontal treatment in patients with periodontitis restored the clinical parameters and periodontal pathogen composition to the levels observed in periodontally healthy individuals. Compared with the PH group, the SPT group exhibited a lower abundance of total bacteria and *Fn* and a higher abundance of *Pg*. This finding correlated with the PI values. The PI values of the SPT group were lower than those of the PH group. Patients undergoing SPT are considered to have better oral hygiene than those in the PH group because oral hygiene is maintained regularly at intervals of 3–6 months. The distribution of periodontal pathogens (except *Fn* and *Pg*) in the SPT group was similar to that in the PH group. *Fn*, a periodontal pathogen, is frequently detected in periodontally healthy individuals. Previous studies have reported that *Fn* was detected at healthy and diseased sites as it could survive in hyperoxic conditions. [34]. *Pg*, a causative agent of periodontitis, is commonly detected in patients with periodontitis. Haffajee et al. [12] reported that the abundance of *Pg* decreased in sites that were successfully treated, but increased at sites with disease recurrence. In this study, the abundance of *Pg* in the SPT group was high, which may be due to patients' previous periodontitis history. Successful periodontal treatment decreased the abundance of *Pg*. However, the abundance of *Pg* in patients who had undergone SPT was higher than that in periodontally healthy individuals, indicating that *Pg* mediates dysbiosis upon changes in the host environment.

In the present study, most of the 9 periodontal pathogens were detected in periodontally healthy individuals at similar levels irrespective of age. This is consistent with the results of Feres et al. [18], who reported that of the 40 species of bacteria examined, there was no significant difference in the frequencies or abundance of most bacteria among different age groups of periodontally healthy individuals. In contrast, the abundance of *Pg* increased with age, whereas that of *Aa* decreased with age in patients with periodontitis [18]. In this study, *Aa* was the least abundant pathogen. The abundance of *Aa* in the PH group was significantly lower than that in PHY group. In contrast, the detection frequencies of *Tf* and *Cr* were significantly higher in the PH group. These differences in the detection frequencies of specific bacteria may be related to differences in age-related changes in systemic immune responses.

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Aged individuals are susceptible to develop chronic inflammatory diseases, such as non-communicable diseases, including cardiovascular diseases, diabetes, chronic obstructive pulmonary disease, rheumatoid arthritis, and cancer [35]. Systemic medications or changes in the immune responses associated with systemic disease can affect the composition and abundance of oral microbes. Additionally, changes in the saliva composition or viscosity with aging may affect the composition and abundance of oral microbes.

We also examined the age-specific distribution of 9 representative periodontal pathogens among the PH and PHY groups. As expected, periodontal pathogens were detected in periodontally healthy individuals. This indicated that the presence of pathogens is not sufficient for the development of periodontal disease and that host immune response and susceptibility are critical for disease development. The disruption of the subgingival microbiota homeostasis results in the development of periodontal disease. The analysis of periodontal pathogen profiles in the PH group revealed the differential distribution of pathogens in patients with periodontitis, which will aid in predicting and diagnosing periodontitis. Additionally, the clinical parameters and microbial profiles of the SPT group were similar to those of the PH group. This suggests that periodic SPT after periodontal treatment enables the restoration of periodontal health and alleviation of dysbiosis. However, *Pg* was significantly more abundant in the SPT group than in the PH group. Hence, the possibility of periodontitis recurrence cannot be ruled out and patients with periodontitis must therefore undergo periodic SPT. The detection frequencies of some pathogens varied according to age in the PH group. Hence, further studies are needed to examine the correlation between age-related changes in the immune system and periodontal bacterial composition.

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