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Comparison of quantitative detection of periodontal pathogens before and after scaling by real-time polymerase chain reaction

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ABSTRACT

Objectives: The purpose of the study is to investigate the quantitative detection of periodontal pathogens before and after scaling by real-time polymerase chain reaction.
Methods: Participants were voluntarily recruited at D university, and saliva samples were extracted before and after scaling. Multiple real-time polymerase chain reactions were used to analyze characteristics and the amount of nine kinds of periodontal pathogens; *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Tannerella forsythia, Treponema denticola, Prevotella intermedia, Fusobacterium nucleatum, Parvimonas micra, Campylobacter rectus,* and *Eikenella corrodens.*
Results: After scaling, most periodontal pathogens except *Eikenella corrodens* were significantly decreased in all subjects($p<0.05$). In addition, the percentage of microorganisms associated with disease, the microorganism risk index of periodontitis and the prevalence of red complex, orange complex, and *Aggregatibacter actinomycetemcomitans* was also significantly reduced after scaling($p<0.05$).
Conclusions: Scaling decreased in the amount of major periodontal pathogens and periodontitis prevalence rate.

Key Words: periodontal pathogens, real-time polymerase chain reaction, scaling

Introduction

Periodontal disease is an infectious disease caused by microorganisms that colonize the tooth surfaces at or below the gingival margins[1,2]. These microorganisms lead to the destruction of the periodontal ligament and alveolar bone that surrounds the teeth, causing loss of attachment to the tooth. When these microorganisms are attached to the tooth surface in microbial communities, they form a layer known as dental plaque[3]. The World Health Organization reports that severe periodontitis occurs in 5-20% of adults worldwide, but milder forms of the disease occurs in approximately 35-50% of the adult population[4]. It is important to confirm the etiology of the disease, to understand the pathogenesis of periodontitis. The detection of periodontal pathogens is strongly influenced by methodological approaches[5].

The overall pattern found in dental plaque development is a very characteristic shift from early predominance of gram-positive, facultative microorganisms to later predominance of gram-negative anaerobic microorganisms, as the plaque mass accumulates and matures[6]. Analysis of these periodontal pathogens is becoming an important aspect of the diagnosis and treatment of periodontal diseases. These diseases are considered polymicrobial diseases that elicit a marked inflammatory response and are associated with members of the indigenous oral microbiota[7]. Among them, *Aggregatibacter actinomycetemcomitans(A. actinomycetemcomitans), Eikenella corrodens(E. corrodens), Fusobacterium nucleatum(F. nucleatum), Porphyromonas gingivalis(P. gingivalis), Prevotella intermedia(P. intermedia), Tannerella forsythia(T. forsythia),* and *Treponema denticola(T. denticola)* should be cited as

major pathogens. *P. gingivalis* and *T. forsythia* are strong markers of periodontitis in adults, and these species have been linked to the progression of the disease[8,9]. The dental plaque presence of members of bacterial species that constitute the red complex, such as *P. gingivalis, T. denticola*, and *T. forsythia*, has been associated with advanced periodontitis and perimplantitis. The presence of orange complex bacteria, such as strains of the species *F. nucleatum* and *P. intermedia*, is associated with periodontitis-related clinical parameters such as pocket probing depth(PPD) and bleeding on probing(BOP)[10]. Bacterial species belonging to the orange complex have been found to precede colonization by species of the red complex[10]. Moreover, the increased prevalence, proportion of bacteria in deep periodontal pockets when compared to moderate or shallow pockets have been reported for both complexes species[10,11]. Recently, strains of the species *A. actinomycetemcomitans* were claimed to be among the most important etiological bacteria involved in aggressive forms of periodontitis due to their ability to produce a powerful leukotoxin[12].

Mechanical removal of subgingival plaque(scaling) is a standard treatment for periodontal diseases[13-15]. Cobb[16] reported that scaling and root planing is the first treatment for most periodontal infections and remains an essential part of successful periodontal therapy. Several studies reported that use of these methods alone or with antiseptic yields clinical benefits[17-19]. Knöfler et al.[20] suggested that full mouth scaling and conventional scaling with root planing have similar effects on target periodontal pathogen species. Yang et al.[21] reported that the prevalence and levels of *P. gingivalis* were significantly reduced after scaling and root planing by real-time polymerase chain reaction.

Quantification of bacteria is the preferred technique to achieve this task nowadays, and real-time polymerase chain reaction(PCR) that relies on real-time detection of the levels of fluorescence generated within the reaction, is probably the most accurate technology[22,23]. Kim et al.[24] reported the prevalence of periodontopathic bacteria in 8 to 18 year old subjects using real-time PCR. Lee and Kim[25] analyzed the changes in oral microorganisms before and after oral hygiene management using a phase contrast microscope. Recently, Cho[26] reported on microorganisms related to periodontopathic bacteria in periodontitis patients and healthy people using multiple real-time PCR. However, few studies have performed quantitative analyses of periodontopathic bacteria in adults after scaling. We undertook this investigation aiming to compare the counts and prevalence of major periodontal pathogens before and after scaling using multiple real-time PCR.

Materials and methods

1. Subjects

Participants were voluntarily recruited from the department of dental hygiene at D university in Daegu from September to December, 2014. Thirty healthy female adults were recruited according to the inclusion and exclusion criteria. No subjects with systemic conditions that could contraindicate periodontal probing or undertake the periodontal disease were enrolled in the study. Neither were subjects that had received periodontal treatment or antimicrobial therapy up to three months prior to specimen collection. Written informed consent was obtained from all participants.

2. Methods

2.1. General characteristics

Data collection was completed with a structured self-reported questionnaire, which was pretested to check for clarity, consistency, and acceptability of the questions to respondents. All questionnaires were checked for completeness and internal errors during data collection. The general characteristics consisted of gender, height, and weight, while oral health behavior consisted of perceived oral health status, systemic disease in family, halitosis, gingival bleeding, brushing method, brushing frequency, and use of oral hygiene auxiliaries.

2.2. Scaling and saliva sampling procedures

The subjects received professional dental care, and a prophylaxis with an hand scaler. If subgingival calculus was detected during the procedure, it was removed. Whole saliva was obtained using a gargling solution kit made by Cytogen(Cytogen Inc., Seoul, Korea) in the morning(10:00-11:00 a.m.) before and after scaling. Saliva samples were collected by spitting resting saliva into sterile plastic bottle after gargling for 20 sec. All samples were labeled with the subject's ID, and the pooled material was kept in an ice until processing.

2.3. Multiple real-time polymerase chain reaction(PCR)

The species-specific primers and TaqMan probes for nine periodontopathogenic bacteria*(Aggregatibacter actinomycetemcomitans(A. actinomycetemcomitans))*, Eikenella corrodens*(E. corrodens), Fusobacterium nucleatum(F. nucleatum), Porphyromonas gingivalis(P. gingivalis), Prevotella intermedia(P. intermedia), Tannerella forsythia(T. forsythia), Treponema denticola(T. denticola), Parvimonas micra(P. micra),* and *Campylobacter rectus(C. rectus))* that trigger periodontal disease were analyzed using the ABI 7500 Fast real-time PCR system(Applied Biosystem Co., Life technologies, USA) adapted to published techniques in Cytogen(Cytogen Inc., Seoul, Korea). Clinical specimens were homogenized by vortexing and centrifuged at 12,000 g, at 4℃. After 30 minutes, the supernatant was discarded and the pellet was employed for DNA extraction using a phenol-choloroform method adapted by Geneall biotechnology[27]. All PCR assays were carried out in duplicate. The standard curves were based on triplicates. The target reference strains were precipitated, diluted, and quantified by using a spectrophotometer. The number of genomes was calculated in product size and ribosomal RNA copy number.

3. Statistical analysis

We examined the comparison of the amount of oral microbacteria before and after scaling using Wilcoxon's signed rank test. To compare the amount of microbacteria and their general characteristics, Mann-Whitney U test and Kruskal-Wallis test were conducted. Data were analyzed using SPSS version 19.0(SPSS 19.0 for windows, SPSS Inc., Chicago, IL., USA), with an α level of 0.05 was considered significant.

Table 1. General characteristics and oral health behaviors of study subject

Variables	N(%)
Gender	
Male	0(0)
Female	30(100)
Perceived oral health status	
Healthy	5(16.7)
Fair	21(70.0)
Poor	4(13.3)
Systemic disease in family	
Yes	9(30.0)
No	21(70.0)
Halitosis	
Yes	16(53.3)
No	14(46.7)
Gingival bleeding	
Yes	4(13.3)
No	26(86.7)
Brushing method	
Rolling	27(90.0)
Bass, etc.	3(10.0)
Use of oral hygiene auxiliaries	
Yes	19(63.3)
No	11(36.7)
Brushing frequency*	2.60±0.50
Height(cm)*	158.97±5.39
Weight(kg)*	53.73±7.32
Body mass index(kg/m^2)*	21.26±2.72

*Mean±SD

Results

1. General characteristics

Table 1 presents the general characteristics of the subjects. 100% were female, and most of them had moderate BMI values(21.26±2.72). More than 70% considered their oral health status as healthy, and the majority of the subjects(70%) had no systemic diseases in the family. 53.3% reported the existence of halitosis, and the majority(86.7%) reported no gingival bleeding. The majority used the rolling technique while brushing (90.0%) and the average brushing frequency was 2.6±0.50 times a day. 63.3% of the subjects used oral hygiene auxiliaries.

2. Quantitative detection of periodontal pathogens by multiple using real-time PCR

The mean periodontopathic bacteria amounts are reported in Table 2 and Fig. 1. The mean population of *F. nucleatum*, was highest among the periodontopathic bacteria in all subjects. *A. actinomycetemcomitans*, *P. micra*, and *P. gingivalis* were higher than the other periodontal pathogens investigated. After scaling, eight periodontopathic bacteria showed a significant decrease, with the exception of *E. corrodens*($p<0.05$): *A. actinomycetemcomitans*, from 2.56E+6±1.11E+7 to 6.54E+5±3.47E+6; *P. gingivalis*, from 2.30E+5±6.25E+5 to 1.84E+4±5.13E+4; *T. forsythia*, from 1.73E+4± 4.28E+4 to 1.63E+3±6.33E+3; *T. denticola*, from 3.88E+5±8.74E+5 to 7.78E+4±1.59E+5; *P. intermedia*, from 3.86E+5±7.84E+5 to 7.45E+4±2.48E+5; *F. nucleatum*, from 1.04E+7±1.50E+7 to 3.99E+6±4.22E+6; *P. micra*, from 3.58E+6±9.05E+6 to 4.39E+6±1.09E+6; and C. rectus, from 3.79E+4±1.40E+5 to 0.00±0.00. *E. corrodens*, with a decrease from 2.16E+4±5.19E+4 to 3.01E+4±9.06E+4, did not reach statistical significance($p>0.05$). Nine of the organisms included in the investigation were detected in oral specimens from all subjects studied. *F. nucleatum* was detected in 100% of subjects before and after scaling. *P. micra* was detected in 90% and 77%, and *E. corrodens* was detected in 63% and 50%, respectively. The prevalence after scaling also decreased from 7% to 20% in nine organisms.

The microorganism risk index was lower than those detected for all periodontal pathogens investigated(▽ 13.26±18.29, $p<0.001$)<Table 3>. In the total bacteria amount, no statistical significance was reached. However, the pathogenic bacteria percentage significantly decreased after scaling(▽3.74±5.19, $p<0.001$). We classified the red complex and orange complex bacteria according to the etiology of disease based on classification described by Socransky et al[10]. Especially, statistically significant difference was reached for red complexes, which were detected in lower concentrations in specimens obtained after scaling(0.14±0.43%), for orange complex (2.23±4.69%), for *A. actinomycetemcomitans*(0.64± 1.79%)<Table 3, Fig. 2>. In subjects with gingiva bleeding, periodontal pathogens decreased by 10.53±8.11%($p<0.05$) after scaling<Table 4>. In subjects

Table 2. Microbiological evaluation of pathogens from subjects before and after scaling by real-time PCR Unit: Mean±SD

Bacterial species	Before scaling		After scaling		ΔValue	p-value*
	Copy counts number(copy/ml)	Prevalence (%)	Copy counts number(copy/ml)	Prevalence (%)		
Aa	2.56E+6±1.11E+7	17	6.54E+5±3.47E+6	7	1.91E+6±7.69E+6	0.043
Pg	2.30E+5±6.25E+5	27	1.84E+4±5.13E+4	17	2.13E+5±5.88E+5	0.012
Tf	1.73E+4±4.28E+4	23	1.63E+3±6.33E+3	7	1.55E+4±4.01E+4	0.028
Td	3.88E+5±8.74E+5	47	7.78E+4±1.59E+5	30	3.11E+5±7.90E+5	0.009
Pi	3.86E+5±7.84E+5	47	7.45E+4±2.48E+5	27	3.38E+5±7.24E+5	0.001
Fn	1.04E+7±1.50E+7	100	3.99E+6±4.22E+6	100	6.36E+6±1.38E+7	0.005
Pm	3.58E+6±9.05E+6	90	4.39E+6±1.09E+6	77	3.14E+6±9.04E+6	0.011
Cr	3.79E+4±1.40E+5	17	0.00±0.00	0	3.79E+4±1.40E+5	0.042
Ec	2.16E+4±5.19E+4	63	3.01E+4±9.06E+4	50	8.54E+3±9.23E+4	0.177(n.s.)

*by Wilcoxon's signed rank test at α=0.05, p-values are shown, n.s. not significant

Aa: *Aggregatibacter actinomycetemcomitans*, Pg: *Porphyromonas gingivalis*, Tf: *Tannerella forsythia*, Td: *Treponema denticola*, Pi: *Prevotella intermedia*, Fn: *Fusobacterium nucleatum*, Pm: *Parvimonas micra*, Cr: *Campylobacter rectus*, Ec: *Eikenella corrodens*

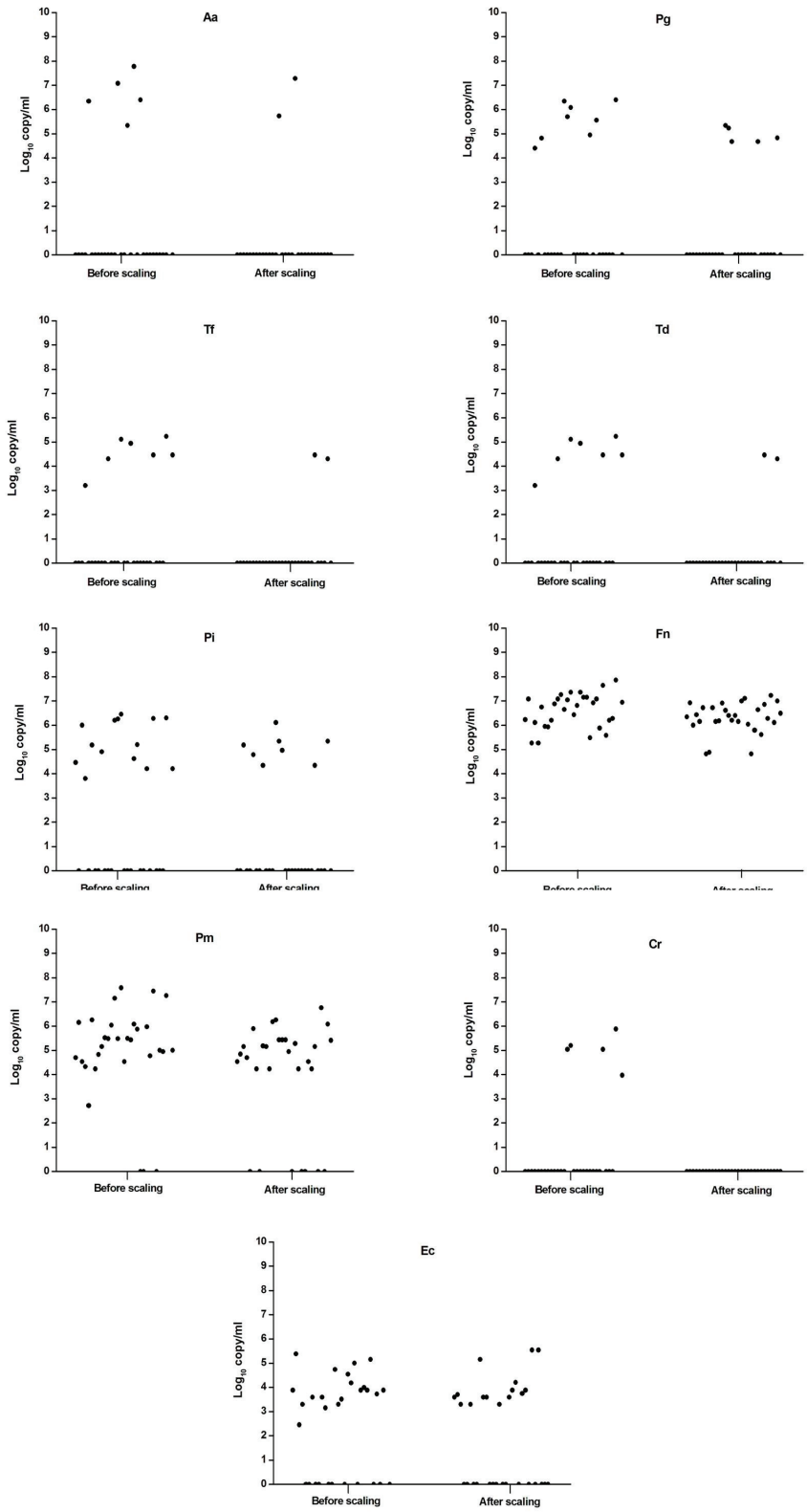

Fig. 1. Comparison of bacterial burdens in subjects before and after scaling by real-time PCR. Number of genomes/ml as indicated in a $\log_{10}$ scale; Same as Table 3 legends.

Table 3. Microbiological evaluation of major periodontopathogens from subjects before and after scaling by real-time PCR

Unit: Mean±SD, copy/ml†

	Before scaling	After scaling	ΔValue	p-value*
Microorganism risk index	23.41±19.34	10.15±5.66	13.26±18.29	<0.001
Total bacteria(copy/ml†)	3.71E+8±4.22E+8	2.99E+8±2.13E+8	7.26E+7±4.70E+8	0.704(n.s.)
%PTGS	5.20±5.47	1.45±1.52	3.74±5.19	<0.001
%RED	0.18±0.45	0.04±0.09	0.14±0.43	<0.001
%ORG	3.66±4.63	1.43±1.27	2.23±4.69	<0.001
%A.a.	0.81±2.45	0.17±0.87	0.64±1.79	<0.001

*by Wilcoxon's signed rank test at α=0.05, p-values are shown, n.s. not significant
†copy/ml: copy count
PTGS: percentage of pathogenic bacteria(microorganisms associated with disease), RED: bacteria of the red complex(Pg: *Porphyromonas gingivalis*, Tf: *Tannerella forsythia*, Td: T*reponema denticola*), ORG: orange complex(Pi: *Prevotella intermedia*, Fn: *Fusobacterium nucleatum*, Pm: *Parvimonas micra*, Cr: *Campylobacter rectus*), A.a.: *Aggregatibacter actinomycetemcomitans*

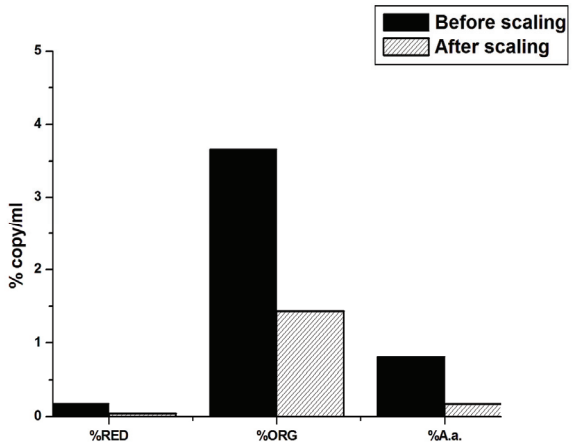

Fig. 2. Comparison of major periodontopathogens in subjects before and after scaling by real-time PCR. Number of genomes of target genes/ml(as indicated in a log_{10} scale)

Table 4. Percentage of pathogenic bacteria(PTGs) according to gingival bleeding from subjects before and after scaling by real-time PCR

Unit: Mean±SD, copy/ml†

Variables	N(%)	ΔValue	p-value*
Gingival bleeding			
Yes	4(13.3)	10.53±8.11	0.020
No	26(86.7)	2.70±3.85	

*by Mann-Whitney U test at α=0.05
†copy/ml: copy count

Table 5. Microorganism risk index according to perceived oral health status from subjects before and after scaling by real-time PCR

Unit: Mean±SD, copy/ml†

Variables	N(%)	ΔValue	p-value*
Perceived oral health status			
Healthy	5(16.7)	9.40±7.10	0.038
Fair	21(70.0)	8.82±7.94	
Poor	4(13.3)	41.36±39.16	

*by Kruskal-Wallis test at α=0.05
†copy/ml: copy count

with poor perceived oral health status, periodontal pathogens decreased by 41.36±39.16% after scaling ($p<0.05$)<Table 5>.

Discussion

This study provides the first empirical evidence in adult women that scaling negatively affects the prevalence of periodontitis by altering the amount of major periodontal pathogens using multiple real-time PCR.

A detection method should be useful, ideally leading to a choice of treatment, which should be simple and aimed at identifying certain forms of periodontal disease associated with the predominant periodontal pathogens, which possess few common characteristics. In this study, we used real-time PCR assays, which are rapid and sensitive techniques for detecting or quantifying microorganisms and for identifying genes or mutations in pathogens associated with antimicrobial resistance. New advances in real-time PCR in clinics will insure better management of patients, and could impact the spread of antibiotic resistance as reported in several studies[22-24,26].

The presence of systemic humoral and cellular reactivity to a variety of oral microorganisms has been found in subjects with different forms of periodontal disease[6-9]. Some investigations have shown the efficacy of scaling in the treatment of gingivities and periodontitis[17-21]. Indeed, scaling and plaque control have been shown to be as effective as surgical modalities in the treatment of periodontal disease. However, no evidence suggests a relationship between scaling and specific microoganism. In this study, we confirmed that scaling has an effect on microflora control using quantitative changes. As shown in Table 2. *A. actinomycetemcomitans*, *P. gingivalis*, and T. *forsythia* are the major periodontopathic bacteria. Here, we found that these major periodontopathic bacteria remarkably decreased after scaling, with the exception of *E. corrodens*($p<0.05$). It is now acknowledged that red complex, the pathogenetic microorganisms implicated in periodontal diseases related to the presence of periodontal pockets and gingival bleeding, orange complex, the pathogenetic microorganisms that are closely related to the red complex in the periodontal pocket region, and *A. actinomycetemcomitans*, the pathogenetic microorganisms that are closely related to the orange complex in the periodontal pocket region[10-12]. Kim et al.[24] reported that the plaque and gingival index have a significant relationship with the counts of *F. nucleatum*, but the counts of *T. forsythia* has a strong relationship with the amount of *T. denticola* and *A. actinomycetemcomitans*, which is confirmed in this study. Our results were also similar to those reported by Könönen and Müller[28]. Kim et al.[29] reported that there was a high level of *P. intermedia* in the moderated pocket(4-5 mm) depth and in the deep pocket(>6 mm) depth sites, and speculated that *P. intermedia* may play an important role in the pathogenesis of periodontal disease. In this study, the amounts and prevalence of red complex(*P. gingivalis*, T. *forsythia*, *T. denticola*), orange complex(*P. intermedia*, *F. nucleatum*, *P. micra*, *C. rectus*) and *A. actinomycetemcomitans* significantly decreased after scaling. These results showed that scaling could effectively reduce the prevalence of gingival disease. Gingival bleeding is closely associated with periodontal disease severity. As shown in Table 4 and 5, gingival bleeding was associated with periodontal pathogens, and scaling statistically significantly decreased the number of these pathogens($p<0.05$). In subject with poor perceived oral health status, periodontal pathogens decreased significantly($p<0.05$) after scaling.

Our study has some methodological limitations. First, our sample size was relatively small so the statistical power may be low. However, we found that scaling affect the amount of periodontopathic bacteria presence and changed the prevalence. Second, this study could not find a relationship between oral health index and amount of oral microbacteria. Further studies with larger samples are necessary to obtain marked and consistent results on this topic. In conclusion, this is the first report on the quantitative evaluation of periodontal pathogens in adult women showing that scaling negatively affects the prevalence of periodontitis by altering the amount of major periodontal pathogens using multiple real-time PCR. Further studies aiming to confirm bacterial changes over the course of periodontitis in each person are needed, and will be helpful for better understanding of periodontal disease pathogenesis. Conclusively, these results can be used to provide customized oral preventive care and treatments.

Conclusions

The results of the study are summarized below. The results suggest that scaling may lead to a decrease in the amount of major periodontal pathogens, which can negatively affect the prevalence of periodontitis. Real-time PCR is a potential diagnostic tool that could be employed.

1. After scaling, most periodontal pathogens, with the exception of *E. corrodens*, decreased significantly in all subjects($p<0.05$).
2. In addition, the percentage of microorganisms associated with disease, the microorganism risk index of periodontitis and the prevalence of the red complex, orange complex, and *A. actinomycetemcomitans* also significantly reduced($p<0.05$).
3. These results could also be used to provide customized oral preventive care and treatments.

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