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Short Communication

Analysis of oral microorganism diversity in healthy individuals before and after chewing areca nuts using PCR-denatured gradient gel electrophoresis

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ABSTRACT

To analyze oral microbial diversity in the saliva of 8 healthy individuals before and after chewing areca nuts. Saliva samples were collected before chewing areca nuts, after chewing areca nuts for 5 min and after chewing areca nuts for 30 min. DNA was extracted, and microbial diversity was examined using PCR-denaturing gradient gel electrophoresis (PCR-DGGE). When examining DGGE profiles collectively, the bands associated with *Streptococcus* and *Veillonella* were the most intense, making them the most prevalent bacteria. Furthermore, the band intensities did not decrease after chewing areca nuts for 5 or 30 min; thus, these bacteria were unaffected. However, when examining some individuals, the band intensities for *Streptococcus* and *Veillonella* became more intense after 5 min of chewing and then returned to the pre-chewing level. This difference may be attributed to the mechanical movements of the oral cavity or individual differences. Other bacteria, such as *Neisseria*, *Actinomycetes*, and *Rothia dentocariosa*, were also found to have an increased or decreased prevalence following areca nut-chewing. Since the predominant species that are present following areca nut-chewing include *Streptococcus* and *Veillonella*, it would seem likely that these bacteria play an important role in the periodontal diseases associated with areca chewing.

1. Introduction

Areca catechu is an evergreen tree that is native to Malaysia and is distributed in tropical regions such as Yunnan, Hainan and Taiwan in China (Nanjing Pharmaceutical College, 1960). The areca nut is widely cultivated in the tropics of Asia and is utilized as a Chinese herbal medicine and for recreational chewing. In ancient China, the areca nut served as one of the 4 major southern medicines (Chinese Pharmacopoeia Committee, 2015) and possesses deworming effects, acts as a destructive narcotic, and has physiological effects on hydration. In modern China, edible areca nuts are mainly divided into 2 types, ones that can be consumed raw and ones that are processed prior to consumption. Due to the ease of chewing, the areca nut has gained popularity, despite its health risks. The regular chewing of areca nuts contributes to oral diseases, such as oral submucous fibrosis, oral squamous cell carcinoma, oral leukoplakia, dental caries, and periodontal diseases (Khan et al., 2012).

The oral cavity is densely populated with many different species of microbes, including predominantly bacteria but also fungi and viruses. Over 200 microorganisms have been identified within the human mouth, including more than 40 species of bacteria. These bacteria include *Streptococcus*, *Staphylococcus*, *Micrococcus*,

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Veillonella, *Neisseria*, *Lactobacillus*, *Actinomycetes*, and *Rothia*, with *Streptococcus* being the most predominant genus within the oral microecosystem. When a person chews areca nuts, the microorganisms attached to the inner wall of the oral cavity are repeatedly rubbed against the oral mucosa due to the component of the areca nut, thereby causing a change in oral microbial quantities and contributing to fibrosis. Areca nut-chewing is associated with oral submucosal fibrosis, a chronic disease that can invade any part of the mouth. This disease is associated with the degeneration of fibrous tissue in the lamina propria and atrophy of the epithelium. The mucous membrane becomes hardened and cords form, eventually causing trismus and impeding the functions of the oral cavity, to ultimately culminate in a precancerous state (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. 2004; Ko et al., 2010; Trivedy et al., 2002). Furthermore, extensive areca nut-chewing promotes the formation of oral mucosal lesions due to alterations in cytokine and proinflammatory cytokine levels.

To better prevent and treat oral diseases associated with the irreversible damage caused by chewing areca nuts, it is important to characterize oral microbial diversity. With the continuous development of molecular platforms, the ability to study the diversity of oral microorganisms has greatly expanded. One such platform is polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE). The basic principle of DGGE is to change the electrophoretic mobility based on differences in DNA chains, concentrations and base compositions in different concentrations of denaturant. This enables DNA fragments of the same size, but with different base compositions, to be separated (Gast et al., 2001). In this study, PCR-DGGE was utilized to examine the oral bacterial composition in the saliva of healthy individuals before and after chewing areca to better characterize its effect on this microecosystem.

2. Materials and methods

2.1. Areca nuts

Areca nuts were purchased from a nut-processing enterprise in Hunan, China.

2.2. Subject selection criteria

Eight healthy adults (21 to 25 years old; 4 men and 4 women) were selected from the College of Food Science and Technology at Hunan Agricultural University. The subjects had not previously chewed areca nuts, had not been administered antibiotics within 3 months of the study, and had no previous history of smoking, systemic diseases, oral ulcers, or an oral local trauma operation.

2.3. Saliva sample collection

Saliva samples were collected prior to areca nut chewing, with all subjects gargling 0.85% sterile saline for 1 to 2 min prior to collection. The saliva was naturally extracted from each of the subjects (2 mL) and labeled as Qa (individual 1)–Qh (individual 8). Similarly, saliva samples were obtained after chewing areca nuts. Each person rinsed their mouth lightly for 1 to 2 min prior to chewing and then chewed half of an areca nut (7 to 10 g) until no sweetness was observed and the residue was broken and fibrous. Samples were collected after 5 or 30 min of chewing and were marked as Ha_5, Ha_{30}, and Hb_5 (individual 1); Hb_{30}, Hc_5, and Hc_{30} (individual 2); etc. Samples were stored at −20 °C until further use.

2.4. DNA extraction and PCR amplification

DNA was extracted from each sample using a GV-Bacterial Genomic DNA Extraction Kit (DingGuo Changsheng biotechnology Co., Ltd, Beijing, China). DNA integrity, purity, fragment size and concentration were determined via 1% agarose gel electrophoresis. The V4 region was amplified using 16S rDNA universal primers, HAD-1 (5′-GACTCCTACGGGAGGCAGCAGT-3′) and HAD-2 (5′-GTATTACCG CGGCTGCTGGCAC-3′), for oral microorganism (Walter J et al., 2000). The PCR amplification consisted of 1 cycle at 94 °C for 5 min, followed by 40 cycles at 98 °C for 10 s, 46 °C for 15 s, and 72 °C for 1 min. After completion, 2% agarose gel electrophoresis was utilized and the target fragments were recovered using a gel recovery kit (Qiagen Inc., Germany).

2.5. Denaturing gradient gel electrophoresis and fingerprint analysis

Formulations were prepared on ice at 30% and 70% gel with proportions of 100% and 0 denaturant solutions. This range is an exact match to the range of the 16S rDNA V4 region of most oral microbes. Following PCR amplification, the obtained amplicons were subjected to DGGE electrophoresis, with a DGGE map constructed and the number of bands recorded and analyzed.

2.6. Denaturing gradient gel electrophoresis band recovery, cloning and sequencing

Based on the DGGE band map, differential banding between samples was visually noted and specific bands were excised. The recovered bands were placed in 2 mL sterilized EP tubes with 0.5 mL of ultrapure water and centrifuged at 16,050 × g for 1 min at 4 °C. This procedure was repeated 2 or 3 times before adding 50 μL of ultrapure water and incubated overnight at 4 °C. On the next day, the recovery tape was removed and placed in a centrifuge at 4,012 × g for 10 min at 4 °C. Next, a portion of the supernatant (10 μL) was then taken for PCR amplification. The amplification was performed as described above and the primers were GC-free. The original primers, HAD-1 and HAD-2, were verified via 1% agarose gel electrophoresis to confirm band size and mass. The obtained amplicon (4 μL) was then cloned into a pClone007-T kit (Qingke Biotech Co., Ltd., Hunan, China), transfected into 100 μL of *Escherichia coli* DH5α competent cells and incubated at 37 °C for 1 h in 300 μL sterile Luria-Bertani (lysogeny broth) medium without antibiotics. The samples were then cultured overnight at 37 °C following supplementation with 100 mg/L ampicillin. Clones were selected at random and colony PCR was performed with the HAD-1 and HAD-2 primers. Positive clones were then further confirmed by sequencing (Qingke Biotech Co., Ltd., Hunan, China). The obtained sequencing results were then compared with the NCBI GenBank database.

3. Results

3.1. Denaturing gradient gel electrophoresis profile analysis, cloning and sequencing

Total DNA was obtained from the 24 samples and amplified using HAD-1 and HAD-2 primers. The generated amplicons were 200 bp in length and were utilized for DGGE electrophoresis analysis. The results showed that there were differences in band distributions between the same individuals before and after chewing and between different individuals. Within the DGGE map, differences in brightness were noted for the 24 samples, to include 15 (Qa, lane 1), 13 (Ha_5, lane 2), and 15 (Ha_{30}, lane 3) distinct signals

for individual 1; 12 (Qb, lane 4), 11 (Hb_5, lane 5), and 10 (Hb_{30}, lane 6) distinct signals for individual 2; 16 (Qc, lane 7), 12 (Hc_5, lane 8), and 11 (Hc_{30}, lane 9) distinct signals for individual 3; 6 (Qd, lane 10), 11 (Hd_5, lane 11), and 8 (Hd_{30}, lane 12) distinct signals for individual 4; 10 (Qe, lane 13), 10 (He_5, lane 14), and 9 (He_{30}, lane 15) distinct signals for individual 5; 8 (Qf, lane 16), 5 (Hf_5, lane 17), and 7 (Hf_{30}, lane 18) distinct signals for individual 6; 10 (Qg, lane 19), 7 (Hg_5, lane 20), and 9 (Hg_{30}, lane 21) distinct signals for individual 7; 11 (Qh, lane 22), 8 (Hh_5, lane 23) and 7 (Hh_{30}, lane 24) distinct signals for individual 8 (Fig. 1). The number of bands noted for each sample reflects the degree of microbial diversity within the microenvironment.

The DGGE map was then further analyzed using Quantity One and Bio-Dap software (http://www.bio-rad.com/) and Shannon, Simpson, Chao1, and ACE indexes were calculated. The bacterial community abundances were determined using the Chao1 and ACE indexes, with a higher value indicating higher specie richness. The Shannon and Simpson indexes were utilized to reflect the degree of bacterial community diversity, with higher values indicating a higher diversity. When examining the Chao1 and ACE indexes (Table 1) for individuals that chewed for 5 verses 30 min, individuals 1, 4 and 7 showed a gradually decrease with time, thus indicating a decrease in oral flora richness. However, in individuals 3, 5, and 6, a gradual increase was noted with an increased chewing time, thus indicating an increased oral flora richness. Individual 2 had increased Chao1 and ACE indexes after chewing for 5 min, but decreased values after chewing for 30 min. Individual 8 was the exact opposite, with lower indexes after 5 min, but higher indexes after 30 min. Since the oral cavity is not in a static state during the collection of saliva and individual differences can be significant, these factors can contribute to diversity index differences between samples. When examining the degree of bacterial community diversity, the Shannon and Simpson index values showed little difference before chewing areca nuts, at 5 min post-chewing or at 30 min post-chewing. These findings suggest that the bacterial community may not be significantly impacted with only a short exposure time.

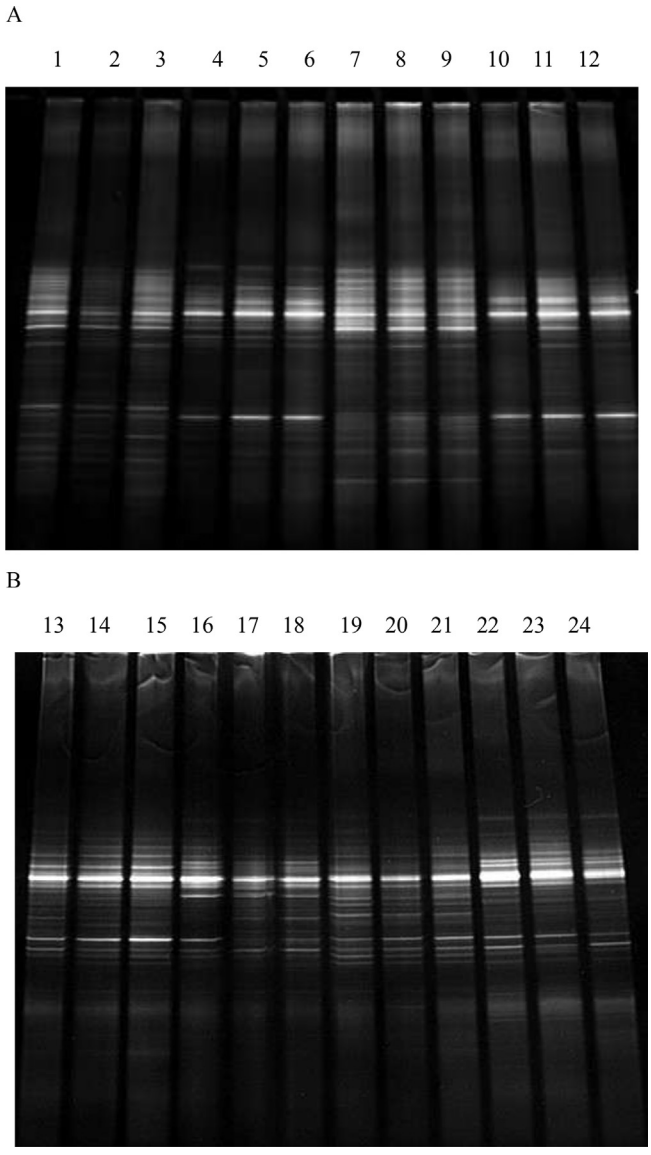

Fig. 1. Denaturing gradient gel electrophoresis (DGGE) electrophoresis of 16S rDNA amplification products from the samples. All samples were detected by DGGE experiments. Fig. 1A represents DGGE profiles of individual 1, individual 2, individual 3, and individual 4. Fig. 1B represents DGGE profiles of individual 5, individual 6, individual 7, and individual 8. Each lane in the DGGE map represents a different state of chewing betel, where lane 1, 4, 7, 10, 13, 16, 19, 22 represent the state before chewing betel nut, lane 2, 5, 8, 11, 14, 17, 20, 23 represent the state of chewing betel after 5 min, lane 3, 6, 9, 12, 15, 18, 21, 24 represent the status after 30 min of chewing betel nut.

A total of 36 bands of varying intensities were excised and positive clones were sequenced (Fig. 2, Table 2). Bands 1, 3, 5, 7, 10, 13, 15, 17, 19, 22, 25, and 29 were commonly distributed and were associated with *Veillonella*. The remaining 12 bands had significantly higher intensities, thus indicating higher prevalence. When comparing individual samples prior to chewing and after chewing areca nuts, little difference in bacterial quantities were noted. In individuals 1 and 3, bands 2, 4, 6, 16, 18, and 20 were commonly distributed and were associated with *Streptococcus* (Fig. 2A, C). In individual 3, bands 16, 18, and 20 had a higher intensity than bands 2, 4, and 6, thus indicating a more prevalent *Streptococcus* genus. Bands 8, 11, 14, 23, 27, and 30 were also commonly positioned and were associated with *Actinomyces*, but a more prevalent genus was not noted. Similarly, bands 9, 12, 21, 24, and 28 were found to be associated with *Neisseria*, with bands 9 and 12 being slightly less intense in individual 2 (Fig. 2B).

Table 1
The statistical table of Alpha diversity index.

Individual	Sample	Observed species	Shannon index	Simpson index	Chao1 index	ACE index
1	Qa	2,963	7.277	0.964	4,486.28	4,640.619
	Ha_5	3,379	8.59	0.982	3,944.533	4,056.448
	Ha_{30}	416	5.929	0.967	545.894	543.754
2	Qb	2,060	5.701	0.92	3,087.132	3,336.075
	Hb_5	2,272	5.636	0.903	3,658.187	3,859.415
	Hb_{30}	2,019	5.572	0.905	3,209.467	3,348.733
3	Qc	1,591	4.88	0.886	2,213.807	2,403.752
	Hc_5	2,145	5.774	0.929	3,331.461	3,600.097
	Hc_{30}	2,667	6.498	0.946	4,225.752	4,515.864
4	Qd	3,338	7.98	0.969	4,797.792	5,020.334
	Hd_5	2,526	7.16	0.968	3,651.621	3,835.659
	Hd_{30}	3,108	8.87	0.989	3,605.485	3,687.375
5	Qe	2,251	5.437	0.899	3,619.757	3,910.279
	He_5	3,004	7.064	0.956	4,307.657	4,428.209
	He_{30}	3,130	6.952	0.955	4,642.524	4,929.062
6	Qf	484	5.256	0.918	657.208	650.811
	Hf_5	2,451	7.23	0.963	2,961.559	3,110.176
	Hf_{30}	2,615	7.147	0.957	3,622.029	3,810.965
7	Qg	2,927	7.68	0.976	4,128.327	4,384.696
	Hg_5	3,177	9.717	0.996	3,545.893	3,567.646
	Hg_{30}	2,560	8.108	0.983	2,947.274	3,077.231
8	Qh	2,929	6.67	0.913	4,096.063	4,287.125
	Hh_5	329	4.735	0.915	479.405	4,61.453
	Hh_{30}	2,928	7.12	0.953	4,152.094	4,351.455

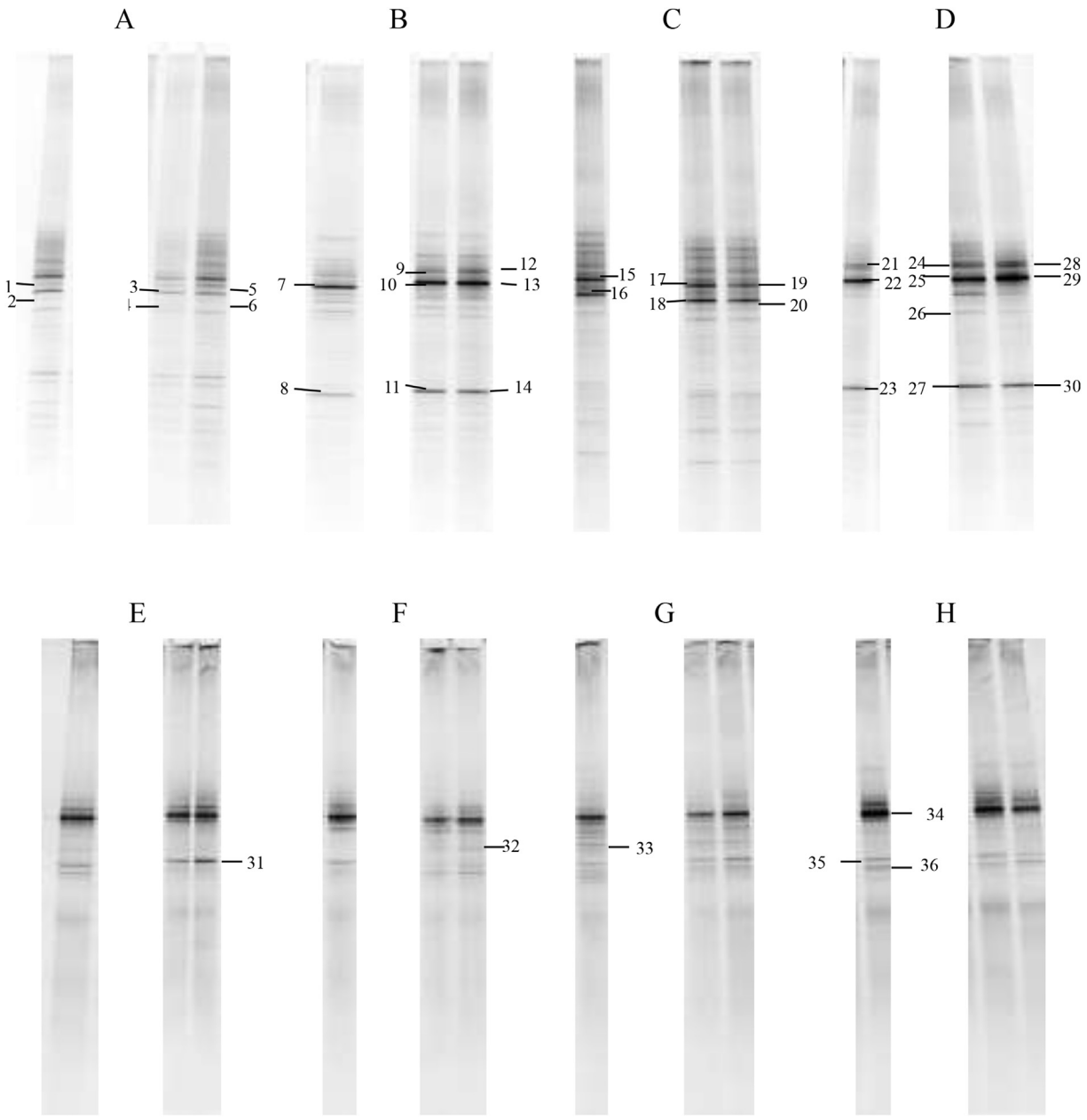

Fig. 2. Distribution patterns of main denaturing gradient gel electrophoresis (DGGE) bands in 8 different individuals. Among all DGGE bands, the main 36 bands were selected. Each strip has a change in brightness. Each of the 3 lanes represents one individual, which is individual 1 (A), individual 2 (B), individual 3 (C), individual 4 (D), individual 5 (E), individual 6 (F), individual 7 (G), individual 8 (H).

In individual 4, band 26 was associated with *Rothia mucilaginosa* (Fig. 2D). In this individual, this band was not detected prior to chewing areca nuts, but after chewing for 5 min, the bacterium was detected. While this strain was present in other individuals, the bands were very faint. The *Neisseria* bands 21, 24, and 28 became fainter after chewing areca nuts for 5 or 30 min. In individual 1, *Veillonella* and *Streptococcus* were the predominant bacteria, with their bands also becoming fainter after 5 min of chewing and then returning to the same intensity prior to chewing after chewing for 30 min. In individual 2, *Neisseria* was faintly detectable prior to chewing, but gained in intensity after chewing; thus suggesting an increase in bacterial numbers.

In individuals 5 to 8, band 34, which is associated with *Streptococcus*, had a significantly higher intensity than the other bands (Fig. 2E to H), and the intensity was not affected by the length of chewing. In individuals 5 and 8, bands 31 and 36 were associated with *Rothia mucilaginosa*, with these bands being less intense in individual 5 prior to chewed areca nuts. In these individuals, the maximum band intensity was noted after chewing for 30 min. In individual 8, the band 36 intensity weakened after 5 min of chewing and returned to the intensity levels noted prior to chewing after 30 min. In individual 6, band 32 was only detected after chewing and was associated with *Phenylobacterium*, thus suggesting that this bacterium is an exogenous species brought in by the areca nut itself. In individuals 7 and 8, bands 33 and 35 were associated with *Actinomyces* and *Rothia dentocariosa*. With chewing, the band intensities decreased, with the largest decrease seen after chewing for 30 min; thus suggesting a decrease in the numbers of these microbes.

Table 2
NCBI BLAST of the main bands from DGGE.

Band No.	Similar bacteria	Similarity, %	Sequence number
1	*Veillonella*	100	NR_113570.1
2	*Streptococcus*	100	NR_117719.1
3	*Veillonella*	100	NR_113570.1
4	*Streptococcus*	100	NR_117719.1
5	*Veillonella*	100	NR_113570.1
6	*Streptococcus*	100	NR_117719.1
7	*Veillonella*	100	NR_113570.1
8	*Actinomyces*	99	NR_029286.1
9	*Neisseria*	99	NR_041989.1
10	*Veillonella*	100	NR_113570.1
11	*Actinomyces*	99	NR_029286.1
12	*Neisseria*	99	NR_041989.1
13	*Veillonella*	100	NR_113570.1
14	*Actinomyces*	99	NR_029286.1
15	*Veillonella*	100	NR_113570.1
16	*Streptococcus*	100	NR_117719.1
17	*Veillonella*	100	NR_113570.1
18	*Streptococcus*	100	NR_117719.1
19	*Veillonella*	100	NR_113570.1
20	*Streptococcus*	100	NR_117719.1
21	*Neisseria*	99	NR_041989.1
22	*Veillonella*	100	NR_113570.1
23	*Actinomyces*	99	NR_029286.1
24	*Neisseria*	99	NR_041989.1
25	*Veillonella*	100	NR_113570.1
26	*Rothia mucilaginosa*	100	NR_044873.1
27	*Actinomyces*	99	NR_029286.1
28	*Neisseria*	99	NR_041989.1
29	*Veillonella*	100	NR_113570.1
30	*Actinomyces*	99	NR_029286.1
31	*Rothia mucilaginosa*	99	NR_044873.1
32	*Phenylobacterium*	99	NR_114055.1
33	*Actinomyces*	97	NR_029286.1
34	*Streptococcus*	100	NR_117719.1
35	*Rothia dentocariosa*	99	NR_044712.2
36	*Rothia mucilaginosa*	99	NR_044873.1

4. Discussion

Veillonella, *Streptococcus*, and *Actinomycetes* are highly abundant strains in the human mouth that can contribute to caries or periodontitis (Peterson et al., 2013; Fan et al., 2008). When chewing areca nuts for a long period of time, the microorganisms in the oral cavity, such as *Veillonella* and *Streptococcus*, produce a large amount of acid. Once the ability of the saliva to buffer this acidity is exceeded, the endogenous acid-producing microorganisms become prominent and can contribute to the development of oral diseases, such as dental cavities or periodontitis (Wang et al., 2017). Thus, chewing areca nuts can contribute to physical damage of the oral cavity wall and influence microbial diversity due to its toxic properties.

After chewing areca nuts for 5 min, the oral cavity responds quickly. The nut alkaloids, such as arecoline, exert certain toxic effects and impact bacterial numbers. Additionally, during chewing, the normal flora attached to the inner wall of the oral cavity are continuously rubbed due to the mechanical movement associated with chewing; thus, bacteria can be sloughed off and numbers decreased. While homeostasis is affected after chewing for only 5 min, homeostasis was regained by 30 min post-chewing, and oral bacterial numbers were increased. *Streptococcus*, *Veillonella* and *Neisseria* were found to be the predominant bacterial genera within the oral cavity. Therefore, these bacteria are presumably important in maintaining oral homeostasis and mediating host interactions with the microenvironment.

When chewing areca nuts, the teeth that are continuously reciprocally rubbed by the nuts and the gums become loosened, red and swollen, thus resulting in periodontal inflammation. When exposed to foods with a high sweetness, like the areca nut, for only a short period of time, the acid production capacity of microorganisms in the oral cavity increases, as does the formation of dental plaque. This increase in acid-fast bacteria will eventually result in the formation of dental caries (Wang et al., 2017; Marsh et al., 2010). In individuals with dental caries, *Streptococcus* species, especially acid-producing ones, are predominant (Peterson et al., 2010; Oda et al., 2015). *Veillonella* is also very common in individuals with oral caries and can adversely affect oral health (Do et al., 2015). While *Veillonella* cannot directly metabolize sugar-producing acids, it can utilize secondary metabolites produced by *Streptococcus*, such as succinic acid, pyruvic acid, and lactic acid, as nutrient sources (Mikx et al., 1975). While *Streptococcus* and *Veillonella* have been implicated in periodontal diseases, such as dental caries, the specific mechanism of their contribution requires further examination.

Similarly, tobacco has also been shown to impact oral microbial diversity and increase the incidences of oral leukoplakia and periodontal diseases (Zonuz et al., 2008). Additionally, studies have shown that chewing tobacco has a significant inhibitory effect on the growth of predominant oral bacteria (Zonuz et al., 2008). However, other studies have found that while nicotine has no significant effect on oral microbial growth (Cogo et al., 2008), tobacco extracts do influence microbial growth (Ji et al., 2014). Furthermore, high concentrations of tobacco leachate were found to inhibit *Streptococcus viridans* and *Candida albicans* growth. However, at lower concentrations, growth is promoted, possibly due to tar being used as carbon and nitrogen sources (Ji et al., 2014). Therefore, just as has been noted in tobacco, the areca nut also possesses substances able to alter the bacterial microflora to ultimately contribute to periodontal diseases.

5. Conclusion

The aim of this study was to examine the effect that chewing areca nuts has on bacterial microflora diversity. Eight healthy individuals without a previous history of areca nut-chewing were examined, with samples collected both before and after chewing for 5 or 30 min. Bacterial diversity was examined using PCR-DGGE. A preliminary analysis of the cloned and subsequently sequenced results showed that chewing did result in a degree of oral microbial alteration in each individual. The PCR-DGGE results showed intensity changes before and after chewing related to *Veillonella*, *Streptococcus*, *Rothia mucilaginosa*, *Neisseria*, and *Actinomyces*, but no significant difference in bacterial numbers were noted. Due to individual differences, the oral cavity may be in a non-static state during chewing, which will directly affect the extraction of oral microbial DNA, thereby affecting subsequent clone sequencing. Furthermore, the diversity indexes showed that an individual's microbial diversity is significantly lowered after chewing areca nuts. While it is clear that areca nuts affect the oral bacterial microflora, the exact mechanisms require further examination.

Conflicts of interest

The authors do not have any conflicts of interest to declare.

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