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DNA Extraction from Low Biomass Plaque Using Two Homogenization Methods for 16S rRNA Sequencing: A Comparative Study

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Conflict of Interest:
All authors declared no conflict of interest.

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Abstract:

Objective:
The composition of subgingival microbiota is related to the status of periodontal health. 16S rRNA sequencing has been proved useful and efficient to assess the composition of subgingival microbiota. However, different homogenization methods utilized to isolate bacterial DNA from subgingival plaque can affect sequencing results, especially from small samples. The study was aimed to compare two common homogenization methods for DNA extraction from plaque samples that allow for accurate genomic sequencing of subgingival microbiota.

Materials and Methods:
Subgingival plaque samples were collected from interproximal sites of molars of one subject and stored in 150 µl TE buffer at -80 ^{0}C. Microbial genomic DNA was extracted using a MO BIO Powersoil DNA Isolation Kit. Cell lysis and homogenization was either performed with 0.1 mm Silica Beads (SI) or 0.7 mm Garnet Beads (GA) on a Vortex. 16S rRNA sequencing (Illumina MiSeq) was then performed to create the corresponding subgingival bacterial genomic profile. Taxonomic assignments to operational taxonomic units (OTUs) from phylum to species level were completed using CLC Genomics Workbench v10 with 98% matching to reference databases (Human Oral Microbiome Database).

Results:
Primary commensal and periodontal bacterial species including *Camphylobacter gracilis, Corynebacterium matruchotii, Fusobacterium nucleatum, and Porphyromonas gingivalis* with relative abundance equal to or more than 1% were identified by sequencing in two groups. There was no significant difference of relative abundance in any species between two groups based on homogenization methods. The alpha diversity (Shannon index, OTU numbers) and beta diversity (Bray-Curtis distance) between two groups were not significantly different. Part of the samples in the SI group could not be sequenced due to failure of amplification.

Conclusion:
Subgingival bacteria were successfully identified by two bead-based homogenization methods. There was no difference of microbial composition and diversity using different homogenization methods.

Introduction

Next-generation sequencing is a very efficient tool to profile microbiota. Specifically, 16S rRNA sequencing is a powerful technique to quantitatively identify the composition of the oral microbiota. The quantity and quality of deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) has to be high to be sequenced. However, sometimes clinical samples may not have sufficient quantity of DNA or RNA for sequencing due to the limited number of bacterial cells. To sequence the subgingival microbiota, it is common to extract DNA from plaque samples collected from multiple sites or teeth to increase the amount of nucleic acid for sequencing [1,2]. However, pooling samples collected from different sites may compromise the characteristics of the individual site given sometimes the periodontal lesion specifically

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exists in a single site. It is important to use the most effective method to extract and purify nucleic acids for profiling subgingival microbiota in a single site with low amount of dental plaque.

There are different methods proposed to optimize the DNA extraction protocol to increase the quantity and quality of nucleic acids [3–6]. These methods include different lysis processes, mechanical treatments [7,8], centrifuge speeds, and elute processes [9]. DNA amplification is also an option when the quantity of DNA from clinical samples is too low [10].

Mechanical homogenization, such as bead beating, is considered as a critical step to destroy cell wall of bacteria to facilitate bacterial nucleic acid extraction [5,11]. Beads are used in several common bacterial DNA isolation kits to facilitate DNA extraction [2,6]. However, the efficacy of different types of beads are rarely compared [4,12]. This study was aimed to compare results of 16S rRNA sequencing using two different bead-based homogenization methods to evaluate the impact of bead-based homogenization on quantity and quality of subgingival bacterial DNA. Additionally, this study assessed the efficacy of the current protocol to extract bacterial DNA from a single site for next generation sequencing.

Materials and Methods

Sample collection

Subgingival plaque samples were collected from one subject to avoid inter-individual variation [6]. This subject diagnosed with gingivitis had good oral hygiene and no probing depth > 4mm. Each sample was collected from one interproximal site of one tooth to prevent pooling microorganisms from different sites. The MoBio PowerSoil® DNA isolation kit (MO BIO Laboratories, Carlsbad, California, USA) was used to extract bacterial DNA from subgingival plaque. It is a commonly used method to extract bacterial DNA from clinical samples [2,6]. This kit originally employs garnet beads, an iron-aluminum silicate, to mechanically rupture bacterial cells to facilitate DNA extraction. For the purpose of this study, two beads, garnet (the GA group) or silica (the SI group), were used. Sample collection sites were paired and matched in the two groups. Sample collection was performed at two time points (6 months apart) to increase sample size.

DNA isolation

The procedures of DNA extraction using a modified MoBio protocol for low biomass samples were done as follows. Plaque samples were collected using a sterile curette (MINI FIVE® GRACEY CURETTE, Hu-Friedy, Chicago, IL, USA) then placed in an eppendorf tube with 150µl TE buffer and stored at -80 ℃ . This volume of TE buffer was then transferred to another tube with 50 µl TE buffer with garnet or silica beads and 200 ul chloroform before extracting DNA. The tubes were horizontally vortexed at maximum speed for 10 minutes on a flat-bed vortex pad with tape. After the homogenization process, cellular debris was removed by centrifugation, and the clean supernatant was transferred to a new tube. PowerSoil solutions C2 and C3 were added and samples were incubated on ice for 5 minutes. Humic debris was removed by centrifugation for 1 minute, and the supernatant was mixed with solution C4 and ethanol. DNA was purified by passing the solution over the PowerSoil column, washing with ethanol and solution C5, as recommended by the manufacturer. DNA was eluted in 60 µl C6 buffer. DNA concentration was measured by spectrophotometer (NanoDrop 2000c, Thermo Fisher Scientific). The DNA was processed for 16S rRNA sequencing through Illumina MiSeq platform (LC Sciences, Houston, TX, USA). The comparison of probing depth or DNA concentration between groups was performed using Student's t test.

16S rRNA gene V4 amplification and sequencing

The 16S rRNA V4 region of bacterial genomic DNA was PCR amplified and sequenced on the Illumina MiSeq platform. Amplification primers contained adapters for MiSeq sequencing and single-index barcodes resulting in PCR products that were pooled and sequenced directly. Read pairs were de-multiplexed based on barcodes and merged by LC Biosciences (Houston, TX, USA). All samples were processed and sequenced together. Clean, merged data was imported into CLC Genomics Workbench v10, with the plug-in Microbial Genomics module. 16S rRNA gene sequences were allocated to specific operational taxonomic units (OTUs) at 98% identity using the Human Oral Microbiome Database (HOMD) [13] OTUs without a match to the HOMD database were given an OTU number, and genus/species identified by BLAST search against the bacterial 16s rRNA database [14].

16S rRNA gene data analysis

Community diversity (alpha and beta diversity) was assessed using the Microbial Genomics Diversity module of CLC Genomics Workbench. OTUs from the abundance table were aligned using MUSCLE with a required minimum abundance of 100. Aligned OTUs were used to construct a phylogenetic tree using Maximum Likelihood Phylogeny

using the Neighbor Joining method and the Jukes Cantor substitution model. Rarefication analysis was done by sub-sampling the OTU abundances in the different samples at a range of depths from 1 to 100,000; the number of different depths sampled was 20, with 100 replicates at each depth. Alpha diversity measures were calculated for observed OTUs and Shannon index and plotted with BoxPlotR (http://boxplot.bio.ed.ac.uk) [15]. Statistical significance in alpha diversity between cohorts (groups of beads or group of teeth for sample collection) was calculated with Student's t-test. PERMANOVA Analysis (Permutational Multivariate Analysis Of Variance) was used to detect significant differences in Beta diversity between groups, and comparisons were visualized using Principal Coordinate Analysis (PCoA). Diversity measures were calculated using Bray-Curtis formula. Differential abundance tests (non-parametric ANOVA) on the OTU frequency table were used to identify significant changes in the relative abundances of individual OTUs between groups. Differential abundance analysis values were calculated for: the max group means (maximum of the average Reads Per Kilobase Million (RPKM's)), -$\log_2$ fold change, standard p-value (significance at less than 0.05), and FDR p-value (false discovery rate adjusted p-value).

Results

Each group had 6 collected plaque samples. The mean DNA concentration was 6.9±4.6 ng/μl in the GA group and 9.1±10.5 ng/μl in the SI group (p=0.65). However, 3 samples in the SI group could not be sequenced due to failure of amplification. The mean probing depth of sites for available samples in each group was 3.17±0.41 mm in GA and 3.00±0.00 mm in SI respectively (p=0.52). There were 293 species identified in these samples. Thirty one species were exclusively identified in the GA group and 31 species were exclusively identified in the SI group. None of bacterial species had significantly different abundance between the GA group and the SI group (adjusted p>0.05). When a less conservative significant level (adjusted p<0.2) was chosen considering the variation of relative abundance and limited sample size, nine species had different abundance between the two bead groups (Table 1). In the species with relative abundance equal to or greater than 1%, eight species (*Porphyromonas gingivalis, Fusobacterium nucleatum subsp. vincentii, Lautropia mirabilis, Fusobacterium HOT 204, Cardiobacterium hominis, Corynebacterium matruchotii, Campylobacter gracilis, Fusobacterium oral taxon 203*) were shared in both GA and SI groups (Table 2).

Regarding the diversity of subgingival microbial community, evenness (Shannon index) was not significantly different between two groups (p=0.33). Richness of microbial community (the number of observed OTUs) was not significantly different between two groups either (p=0.63) (Figures 1A and 1B). The graph of principal coordinated analysis (Bray-Curtis) did not show significant separation of samples in the

Table 1. Comparison of microbial relative abundance using two bead-based homogenization methods (garnet or silica)

Species	Fold change (Log_2)	P-value	Adjusted p-value
Streptococcus intermedius	-5.94	1.85×10^{-4}	0.11
Vibrio parahaemolyticus	-7.16	1.01×10^{-3}	0.12
Aggregatibacter sp. (HOT-898)	-6.37	1.17×10^{-3}	0.12
Parvimonas micra	-4.8	1.35×10^{-3}	0.12
Olsenella uli	-8.64	1.46×10^{-3}	0.12
Prevotella sp. (HOT-526)	-4.94	1.50×10^{-3}	0.12
Corynebacterium durum	-6.45	1.51×10^{-3}	0.12
Prevotella sp. (Strain_P4P_53)	-4.3	2.81×10^{-3}	0.18
SR1 [G-1] sp. (HOT-345)	-5.73	2.83×10^{-3}	0.18

These listed species had relative fold change of abundance between two groups (garnet/silica) with a false discovery rate (FDR) adjusted p-value < 0.2. Minus value of the fold change means the relative abundance in the garnet group is smaller than the silica group.

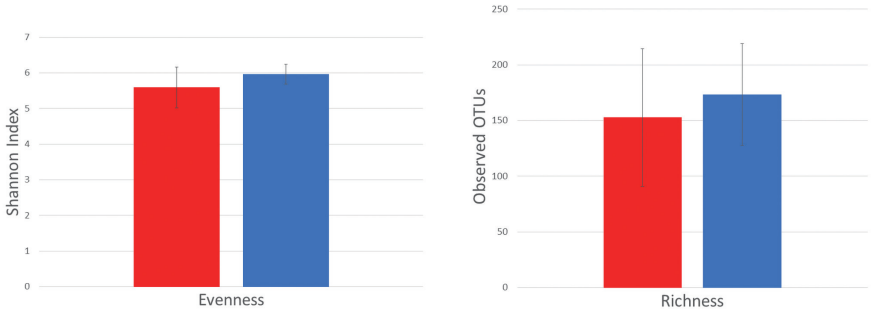

Figure 1. Alpha diversity of microbial community in the garnet group (red) and the silica group (blue). No significant differences between the two groups were observed for (1A) evenness (Shannon index) and (1B) richness (Observed OUT number).

Table 2. Relative abundance of bacterial species

The Garnet Group		The Silica Group	
Bacterial species	**Relative abundance**	**Bacterial species**	**Relative abundance**
Porphyromonas gingivalis	0.07	***Porphyromonas gingivalis***	0.09
Fusobacterium nucleatum_ subsp._vincentii	0.05	***Fusobacterium nucleatum_ subsp._vincentii***	0.08
Lactobacillus crispatus	0.04	***Lautropia mirabilis***	0.03
Lautropia mirabilis	0.03	***Fusobacterium sp._HOT_204***	0.03
Fusobacterium sp._HOT_204	0.03	***Cardiobacterium hominis***	0.03
Neisseria flavescens	0.03	*Prevotella intermedia*	0.03
Fusobacterium nucleatum_ subsp._animalis	0.02	***Corynebacterium matruchotii***	0.02
Fusobacterium sp._oral_ taxon_203	0.02	***Campylobacter gracilis***	0.02
Bacteroidales_[G-2] sp._oral_ taxon_274	0.02	*Porphyromonas endodontalis*	0.02
Neisseria oral_taxon_018	0.02	*Fretibacterium sp._oral_ taxon_358*	0.02
Corynebacterium matruchotii	0.02	*Propionibacterium propionicum*	0.02
Cardiobacterium hominis	0.02	*Treponema sp._oral_ taxon_262*	0.02
Fusobacterium nucleatum_ subsp._polymorphum	0.01	*Leptotrichia hongkongensis*	0.02
Campylobacter gracilis	0.01	***Fusobacterium sp._oral_ taxon_203***	0.01
Tannerella forsythia	0.01	*Fretibacterium fastidiosum*	0.01
Dialister invisus	0.01	*Treponema sp._oral_ taxon_238*	0.01
Alloprevotella tannerae	0.01	*Treponema denticola*	0.01
Ottowia oral_taxon_894	0.01	*Prevotella sp._oral_taxon_526*	0.01

All listed bacterial species had relative abundance ≥ 1%. Bold species were identified in both garnet and silica groups.

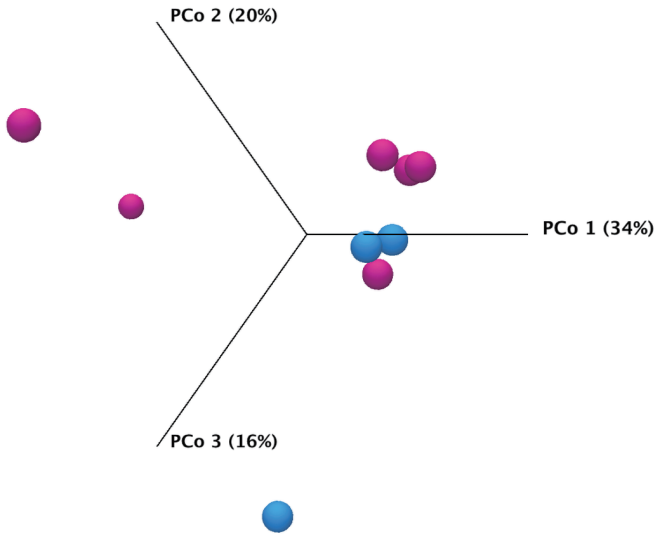

Figure 2. Principal coordinate analysis (PCoA) plot with Bray-Curtis dissimilarity. Microbial communities in the garnet group (red) did not clearly separate from microbial communities in the silica group (blue).

GA group from samples in the SI group (p=0.31) (Figure 2). Taken together with Richness and Evenness data, the results indicated that homogenization methods did not affect the diversity of microbial communities.

Since plaque samples were collected from different molars, analyses were performed to assess the effects of collection sites on microbial abundance and diversity. Only three samples in the GA group were selected for the analyses to match three samples in the SI group. These samples were collected from tooth #46 and tooth #47. Two bacterial species (*Lactobacillus crispatus* and *unnamed species*) had significantly different abundance between two collection sites (#46 and #47; adjusted p=0.02 and 0.03 respectively). However, the Shannon index and OTU numbers were not significantly different between two collection sites (p= 0.87, 0.44 respectively). Beta diversity (Bray-Curtis) of the microbial community did not have significant difference between two collection sites (p=0.73). In contrast, comparing samples collected at the first time point and second time point revealed a significant change in microbial community (p=0.01), which reflects the expected community variation over time (Figure 3).

Discussion

Methods of extracting bacterial DNA that include a bead beating cell lysis step have the advantages of effectively releasing DNA from gram negative bacteria and homogenizing the clinical samples. It has been demonstrated that using DNA isolation methods including bead beating can increase the chance of detecting more species in periodontal microbiota than methods without bead beating [16]. However, bead beating

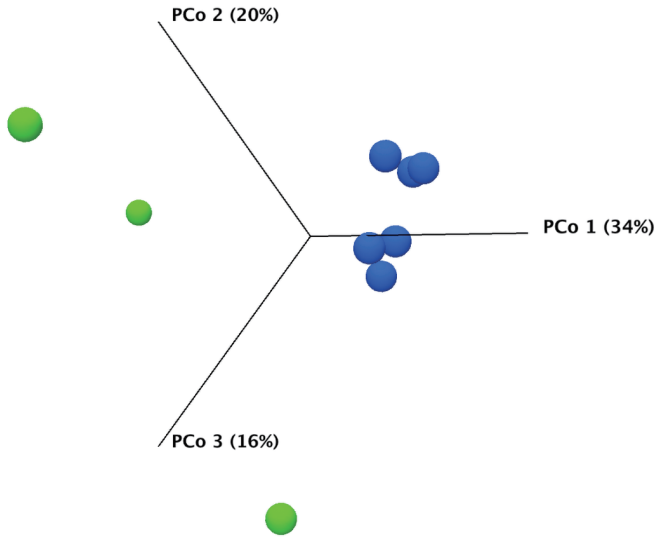

Figure 3. Principal coordinate analysis (PCoA) plot with Bray-Curtis dissimilarity. Microbial communities taken at the first time point (blue) did clearly separate from microbial communities from the second time point (green).

Table 3. Comparison of two bead-based homogenization methods

	Silica Beads (SI)	Garnet Beads (GA)
Size of the beads	0.1 mm	0.7 mm
Cell lysis efficacy	Smaller beads may result in fewer short fragments of DNA than larger beads. However, small beads may not effectively lyse a small number of cells collected from clinical samples.	Larger beads are more likely to efficiently lyse cells than smaller beads. However, large beads may result in shearing the DNA into short fragments that increase the bias of sequencing.
Conclusions	The bead-based homogenization methods do not have significant impact on the results of 16S rRNA sequencing for subgingival plaque samples. However, the chance of having unsequenced samples using silica beads might be a concern.	

may result in shearing the DNA into short fragments [7] and may increase the bias of sequencing [17]. Although MoBio PowerSoil® DNA isolation kit is commonly used [18], the garnet beads with a sharper edge and a larger size than other beads might not be the most appropriate beads for subgingival plaque samples since they may result in unwanted fragments causing bias of sequencing results and it had been shown that beads with a larger size might not be more efficient in DNA extraction than beads with a smaller size [12]. The current results demonstrated different beads did not have significant effects on results of 16S rRNA sequencing. However, it is important to know that three out of six samples in the silica group could not be sequenced. Although the mean DNA concentration of samples in both GA and SI groups was not significantly different, some DNA in these samples might come from mammalian cells. Silica beads might not destroy bacterial cells as effectively as garnet beads, especially for little plaque collected from one site of the tooth in a patient without periodontitis (Table 3).

Current results of this study showed this bacterial DNA isolation protocol is able to efficiently identify both gram positive and gram negative bacteria in oral microbiota. In the identified species with relative abundance $\geq 1\%$, *Lautropia mirabilis* [19–21], *Campylobacter gracilis* [21,22], and *Corynebacterium matruchotii* [23,24], were associated with shallow pocket and periodontal health. *Corynebacterium matruchotii* is the major species in oral plaque and present at significant levels [24].

Although these plaque samples were collected from sites of a patient without periodontitis and without deep probing depth, several periodontal pathogens including *Porphyromonas gingivalis, Tannerella forsythia* and *Treponema denticola* were identified in these samples. These results support the concept that bacterial species in plaque samples collected from healthy sites or diseased sites may have significantly different relative abundance but these species are commonly identified in both healthy and diseased sites [25]. Both periodontal pathogens and commensal bacteria can trigger the inflammatory responses if polymicrobial dysbiosis happens.

It has been demonstrated that the inter-individual variation in microbiota exceeded the variation resulting from choice of the DNA extraction method[6]. Since all samples in this study were collected from the same subject, the inter-individual variation was not a concern. However, the current results showed that the variation caused by the sample collection site and the collection time point appeared to be more notable than the variation caused by the bead-based homogenization meth-

od. It is known that oral microbiota is site-specific and has temporal dynamics [26–29]. These results should be carefully interpreted because of the limited sample size.

Conclusion

The difference of beads using in bacterial DNA extraction and the difference of sample collection sites with a similar clinical condition did not have significant impact on the results of 16S rRNA sequencing. The chance of having unsequenced samples using the silica bead might be a concern.

Acknowledgments

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