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Microbial signatures of oral dysbiosis, periodontitis and edentulism revealed by Gene Meter methodology

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ABSTRACT

Conceptual models suggest that certain microorganisms (e.g., the "red" complex) are indicative of a specific disease state (e.g., periodontitis); however, recent studies have questioned the validity of these models. Here, the abundances of 500+ microbial species were determined in 16 patients with clinical signs of one of the following oral conditions: periodontitis, established caries, edentulism, and oral health. Our goal was to determine if the abundances of certain microorganisms reflect dysbiosis or a specific clinical condition that could be used as a 'signature' for dental research. Microbial abundances were determined by the analysis of 138,718 calibrated probes using Gene Meter methodology. Each 16S rRNA gene was targeted by an average of 194 unique probes (n = 25 nt). The calibration involved diluting pooled gene target samples, hybridizing each dilution to a DNA microarray, and fitting the probe intensities to adsorption models. The fit of the model to the experimental data was used to assess individual and aggregate probe behavior; good fits ($R^2 > 0.90$) were retained for back-calculating microbial abundances from patient samples. The abundance of a gene was determined from the median of all calibrated individual probes or from the calibrated abundance of all aggregated probes. With the exception of genes with low abundances (<2 arbitrary units), the abundances determined by the different calibrations were highly correlated ($r \sim 1.0$). Seventeen genera were classified as 'signatures of dysbiosis' because they had significantly higher abundances in patients with periodontitis and edentulism when contrasted with health. Similarly, 13 genera were classified as 'signatures of periodontitis', and 14 genera were classified as 'signatures of edentulism'. The signatures could be used, individually or in combination, to assess the clinical status of a patient (e.g., evaluating treatments such as antibiotic therapies). Comparisons of the same patient samples revealed high false negatives (45%) for next-generation-sequencing results and low false positives (7%) for Gene Meter results.

1. Introduction

Periodontitis is an inflammatory disease associated with the tissues that support the teeth. The disease causes the progressive loss of bone that could result in tooth loss (Armitage, 1999). A small assemblage of bacterial species was once thought to be the cause of periodontal disease. This assemblage consisted of *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*, and was ominously dubbed the "red complex" (Dewhirst et al., 2010; Kumar et al., 2003; Holt and Ebersole, 2005). The prevailing idea at the time was that the red complex changed the signaling pathways of the host. This change made the host vulnerable and led to a change in the relative abundance of microorganisms thus causing the disease (Darveau, 2010; Hajishengallis et al., 2011). However, members of the red complex have been discovered in people who do not have periodontal disease (Diaz et al., 2006; Bik et al., 2010; Liu et al., 2012). Therefore, members of the red complex could be classified as pathogens or harmless commensals. This fact, along with the finding that the oral microbiome of humans is much more diverse than previously thought (Griffen et al., 2012; Zaura et al., 2009), has prompted a paradigm shift in the understanding of the origins of periodontal disease. This new paradigm states that periodontal disease is not caused by the presence of specific bacteria, but by changes in the population levels of species in the oral microbiome (Hajishengallis and Lamont, 2012). What begins as a symbiosis between host and microbes becomes a dysbiosis as the microorganisms transcend beyond the host-imposed boundaries. The premise of our study is that a change in microbial populations can be determined by the relative abundances of individual species in patients with different clinical conditions.

Oligonucleotide microarray technology has been used to profile microbial communities for quite some time (Huyghe et al., 2008; Iwai et al., 2012; Topcuoglu and Kulekci, 2015; Roth et al., 2004; Pozhitkov et al., 2007). A new version of the methodology, the Gene Meter, was recently introduced (although not explicitly named "Gene Meter" in refs. Pozhitkov et al., 2014a; Harrison et al., 2013). Unlike conventional DNA

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microarrays, a calibration procedure is conducted prior to applying samples onto the microarrays (Hunter et al., 2016a; Pozhitkov et al., 2016a). The calibration procedure establishes the microarray probe responses to increasing target concentrations (i.e., a dilution series). Individual probes are calibrated or, alternatively, groups of probes collectively specific to a target (i.e., aggregates) are calibrated. Both approaches of calibration were compared in this study.

Next generation sequencing (NGS) has also been used to profile oral microbial communities (Diaz et al., 2012; Liu et al., 2012; Nasidze et al., 2009; Ahn et al., 2011; Cephas et al., 2011; Simón-Soro et al., 2013; Xu et al., 2014; Sato et al., 2015). This approach involves the massive sequencing of thousands to millions of DNA strands in a single run (Pozhitkov et al., 2011). While microbial abundances may be quantified by NGS, it remains to be determined if the quantifications truly represent the quantities of the genes in a biological sample. Only a limited number of studies have rigorously challenged their quantitative capabilities. In one study, a complex microbial mixture was spiked with five fungal targets including *Laccaria proxima* and *Metschnikowia noctiluminum* (Amend et al., 2010). It was shown that depending on the target, the coefficient of determination for the relationship between actual quantities of the target and its sequencing counts was very low. In another recent study, NGS is shown to be a semi-quantitative approach for examining fungal populations (Dannemiller et al., 2014). The results of the study showed that 454 pyrosequencing counts are, at best, moderately related with spore concentration measurements by qPCR (R^2 ~ 0.5). An RNA-seq spike-in study has also been conducted (Jiang et al., 2011). Ninety-six RNA sequences of concentrations varying 6 orders of magnitude were mixed and sequenced and the concentrations were correlated to the number of reads. Although the study design did not investigate the response of an individual target to concentration, the results suggest that the RNA-seq sensitivity can vary up to 10-fold depending on the gene target (Jiang et al., 2011). The uncertainty associated with quantification by sequencing raises questions about the validity of such quantifications and warrants a well-controlled calibration study, which was done here.

The objectives of the study are three-fold: (i) to demonstrate the utility of the Gene Meter approach to precisely determine 16S rRNA gene abundances using two calibration approaches, (ii) to determine if certain microorganisms have an abundance signature that can be employed to identify specific or general oral health conditions, and (iii) to compare and contrast two different technologies (Gene Meter and DNA sequencing) using the same patient samples.

2. Materials and methods

The DNA sequencing, the study design, and sample collection methods have been previously published (Pozhitkov et al., 2015). The same amplified DNA used for DNA sequencing was also analyzed by the Gene Meter approach. Splitting the samples in this way enabled direct comparison of results from two distinctly different molecular methods. To familiarize readers with the previous study, we have provided a brief overview of patient recruitment, enrollment, and exclusion criteria, sample collection, DNA extraction and amplification, and DNA sequencing analyses, below.

2.1. Patient recruitment, enrollment, and exclusion criteria

The adult patients were recruited at the University of Washington, Seattle, USA, and the University of Düsseldorf, Germany and enrolled if they had one of the following clinical conditions: severe periodontitis, caries, edentulism, and oral health. A periodontitis case was defined as having at least two interproximal sites at different teeth with clinical attachment loss (CAL) of 6 mm or greater and at least one interproximal site with probing depth (PD) of 5 mm or greater (Page and Eke, 2007) and a minimum of 20 permanent teeth, not including third molars. Patients were excluded from the periodontitis group if they had any established caries lesion or wore a removable partial denture. A caries case was defined as having the following number of teeth with established caries lesions: 6 or more teeth in subjects 20 to 34 years of age; 4 or more teeth in subjects 35 to 49 years of age; and 3 or more teeth in subjects 50 years of age and older. Established caries was defined as a class 4 lesion according to the International Caries Detection and Assessment System. The number of teeth with caries lesion in caries cases was greater than one standard deviation above the mean of caries extent in respective age group the U.S.A. (Dye et al., 2007). Exclusion criteria for a caries case were interproximal sites with CAL of 4 mm or greater or PD of 5 mm or greater (Page and Eke, 2007). An edentulous case had to be completely edentulous in both jaws and their teeth had to be extracted more than one year before the enrollment in the study. A healthy case was defined as having 28 teeth, not counting third molars, or 24 or more teeth, not counting third molars if premolars had been extracted for orthodontic reasons or were congenitally missing with no signs of oral disease. Exclusion criteria for a healthy case included: smoking, loss of permanent teeth due to caries or periodontal disease, any interproximal sites with CAL of four or greater or PD of 5 mm or greater, or any established caries lesions. Exclusion criteria for all groups included: oral mucosal lesions, systemic diseases, and use of antibiotics or local antiseptics within 3 months prior to the study.

2.2. Sample collection, DNA extraction and PCR amplification

For all but the edentulous patients, supra- and subgingival plaque was collected from sites with the deepest probing depth in each sextant. One sterile paper point per site was inserted into the deepest aspect of the periodontal pocket or gingival sulcus. Biofilm from oral mucosae was collected by swiping a sterile cotton swab over the epithelial surfaces of the lip, left and right buccal mucosae, palate, and dorsum of the tongue (Beikler et al., 2006). Samples were stored at −80 °C.

Microbial DNA was isolated from cells by physical and chemical disruption using zirconia/silica beads and phenol-chloroform extraction in a FastPrep-24 bead beater (Flemmig et al., 2012). Prokaryotic 16S rRNA genes were amplified using universal primers (27F and 1392R) using the GemTaq kit from MGQuest (Cat# EP012). The PCR program involved a pre-amplification step of 10 cycles with an annealing temperature of 56 °C followed by 20 amplification cycles with annealing temperature 58 °C. In each cycle, elongation time was 1 min 10 s, at 72 °C. PCR was finalized by extended elongation for 5 min. PCR products were purified with DNA Clean & Concentrator columns (Zymo Research, USA) and quantified using the NanoDrop (Agilent, USA). Equal quantities of PCR product derived from swab and paper point samples were pooled together for each patient. For edentulous patients, there were no paper point samples. Each purified PCR product was sequenced on a Roche 454 Jr. instrument as previously described (Pozhitkov et al., 2015).

2.3. DNA sequencing analysis

The obtained sequences were uploaded to the MG-RAST web server (Meyer et al., 2008). The MG-RAST pipeline assessed the quality of sequences, removed short sequences (multiplication of standard deviation of length cutoff of 2.0) and removed sequences with ambiguous bp (non-ACGT; maximum allowed number of ambiguous base pair was set to 5). The pipeline annotated the sequences and allowed the integration of the data with previous metagenomic and genomic samples. The Ribosomal Database Project (RDP) was used as an annotation source, with minimum sequence identity of 97%, maximum e-value cutoff of 10^{-5}, and minimum sequence length of 100 nt.

2.4. Microarray probe design

Microarray probes were obtained by tiling along 16S rRNA sequences of 597 oral bacteria (Table 1 in reference Hunter et al.,

2016b) using a program written for this purpose. Redundant probes were removed. Each probe was 25 nt in length. The 16S rRNA gene sequences used to design the probes ranged from 424 to 13,214 nt in length with an average of 1492 nt. Three of the 16S rRNA sequences also included 5S and 23S rRNA genes. The microarrays were created by NimbleGen (now Roche Inc.).

2.5. Sample labeling

PCR products (above) were purified using a "DNA Clean & Concentrator" kit (Zymo Research, USA), dried under a flow of dry nitrogen and labeled using ULYSIS direct chemical labeling kit. The attached dye was Alexa Fluor 546 (i.e., spectral analog of Cy3).

Table 1
Bacterial species having significantly different abundances for patients with health versus those with edentulism based on individual probes. n_probes, number of probes used to determine the species abundance; Two-sided *t*-tests were based on four patients with health and four patients with edentulism and alpha = 0.05.

Phylum/class	Genus/species/GI number	n_probes	Abundance (X ± s.d.) by	
			Health	Edentulism
Actinobacteria/Actinobacteria	*Actinomyces meyeri* (1838945)	74	2.4 ± 0.5	5.1 ± 1.7
	Actinomyces odontolyticus (853707)	36	2.2 ± 0.5	4.4 ± 1.4
	Actinomyces odontolyticus strain 20536T (6997974)	31	2.4 ± 0.4	5.5 ± 1.7
	Actinomyces sp. oral strain Hal-1065 (14537929)	168	2.3 ± 0.4	4.7 ± 1.1
Actinobacteria/Coriobacteria	*Atopobium rimae* (10719609)	22	2.7 ± 0.6	6.1 ± 2.1
Bacteroidetes/Bacteroidetes	*Bacteroides ureolyticus* (173919)	387	2.7 ± 0.7	5.6 ± 2.0
	Porphyromonas endodontalis ATCC 35406 (294287)	89	2.7 ± 0.8	5.6 ± 1.9
	Prevotella nigrescens ATCC 33563 (294425)	57	2.4 ± 0.6	5.1 ± 1.8
	Prevotella sp. oral clone AH125 (9988924)	78	2.4 ± 0.6	5.0 ± 1.7
	Prevotella sp. oral clone AO096 (9988919)	98	2.6 ± 0.6	5.4 ± 1.7
	Prevotella sp. oral clone AU069 (9988926)	148	2.6 ± 0.6	5.3 ± 1.8
	Prevotella veroralis ATCC 33779 (294427)	41	2.8 ± 0.8	5.6 ± 1.9
	Tannerella forsythia (289382)	50	2.4 ± 0.6	5.2 ± 1.7
Bacteroidetes/Flavobacteria	*Capnocytophaga* sp. oral clone BU084 (14537978)	65	2.6 ± 0.6	5.5 ± 1.9
Bacteroidetes/Flavobacteria	*Capnocytophaga* sp. oral clone X066 (9988942)	44	2.6 ± 0.7	5.7 ± 1.9
Firmicutes/Bacilli	*Streptococcus anginosus* strain ATCC33397 (4406240)	53	2.5 ± 0.6	5.3 ± 1.7
	Streptococcus salivarius (176047)	28	2.1 ± 0.4	4.5 ± 1.1
	Streptococcus sp. oral clone FO042 (16612189)	6	2.4 ± 0.6	5.5 ± 1.9
	Streptococcus sp. oral clone FP015 (16612190)	61	2.2 ± 0.5	4.9 ± 1.7
	Streptococcus thoraltensis (2578813)	275	2.6 ± 0.7	5.5 ± 1.9
	Lactobacillus paracasei subsp. *paracaseie* (1808583)	79	2.5 ±	5.2 ± 1.7
	Mogibacterium vescum strain:ATCC 700697 (4140242)	83	2.6 ± 0.7	5.6 ± 1.9
Firmicutes/Clostridia	*Catonella morbi* (1067117)	118	2.5 ± 0.6	5.1 ± 1.7
	Eubacterium sp. oral clone BS091 (14537971)	80	2.7 ± 0.8	5.4 ± 1.8
	Eubacterium sp. oral clone EH006 (14537979)	106	2.7 ± 0.7	5.9 ± 2.1
	Eubacterium sp. oral strain A35MT (9837454)	41	2.5 ± 0.7	5.5 ± 1.8
	Peptostreptococcus sp. oral clone BS044 (14537968)	79	2.6 ± 0.6	5.5 ± 1.7
	Selenomonas sp. oral clone EW076 (14537908)	42	2.4 ± 0.6	4.9 ± 1.6
	Selenomonas sp. oral clone EW084 (14537910)	91	2.7 ± 0.7	5.7 ± 2.0
	Selenomonas-like sp. oral strain GAA14 (9837482)	129	2.5 ± 0.6	5.4 ± 1.9
Firmicutes/Cocci	*Staphylococcus saccharolyticus* (576608)	46	2.4 ± 0.6	5.1 ± 1.8
Firmicutes/Erysipelotrichia	*Solobacterium* sp. oral clone K010 (9988916)	113	2.7 ± 0.7	5.5 ± 1.9
Firmicutes/Mollicutes	*Mycoplasma faucium* strain DC333(T) (4883891)	176	2.6 ± 0.7	5.5 ± 1.8
Firmicutes/Negativicutes	*Dialister* sp. oral clone BS095 (9837480)	99	2.6 ± 0.7	5.3 ± 1.7
	Dialister sp. oral strain GBA27 (9837481)	63	2.7 ± 0.7	5.4 ± 1.8
	Megasphaera sp. oral clone BU057 (14537975)	305	2.5 ± 0.7	5.3 ± 1.8
	Veillonella dispar (793883)	40	2.4 ± 0.6	5.1 ± 1.7
Fusobacteria/Fusobacteria	*Leptotrichia* sp. oral clone DR011 (14537926)	215	2.6 ± 0.6	5.2 ± 1.7
	Leptotrichia sp. oral clone FB074 (14537951)	114	2.5 ± 0.6	5.5 ± 1.9
Proteobacteria/Betaproteobacteria	*Comamonas* sp. isolate 158 (3046563)	33	2.6 ± 0.7	5.3 ± 1.7
	Kingella denitrificans (174983)	63	2.6 ± 0.7	5.3 ± 1.7
	Kingella oralis (174982)	184	2.5 ± 0.6	5.3 ± 1.8
	Neisseria sp. oral strain B33KA (10048304)	74	2.7 ± 0.7	5.8 ± 2.0
	Neisseria subflava strain U37 (5327189)	12	2.8 ± 0.9	6.5 ± 1.8
Proteobacteria/Gammaproteobacteria	*Klebsiella oxytoca* (14549203)	65	2.5 ± 0.5	5.5 ± 1.9
	Klebsiella pneumoniae strain ATCC13884T (3282032)	8	2.3 ± 0.6	4.8 ± 1.5
	Pseudomonas aeruginosa strain:ATCC 27853 (6863010)	30	2.7 ± 0.7	5.6 ± 1.9
Spirochaetes/Spirochaetes	*Treponema pectinovorum* 8:A:33768 (3757506)	72	2.6 ± 0.6	5.6 ± 2.0
	Treponema pectinovorum OMZ831 (10764812)	108	2.7 ± 0.8	5.5 ± 1.9
	Treponema socranskii (2653628)	77	2.3 ± 0.5	4.9 ± 1.6
	Treponema sp. I:B:C7 (2586374)	16	2.8 ± 0.8	5.8 ± 1.9
	Treponema sp. I:E:U17A (2586380)	37	2.6 ± 0.7	5.4 ± 1.8
	Treponema sp. I:F:D13 (2586376)	70	2.5 ± 0.6	5.1 ± 1.7
	Treponema sp. I:K:T3 (2586382)	35	2.6 ± 0.6	5.5 ± 1.8
	Treponema sp. I:N:D47 (2586384)	113	2.7 ± 0.7	5.7 ± 1.9
	Treponema sp. I:O:AF16 (3046569)	43	2.5 ± 0.6	5.4 ± 1.9
	Treponema sp. I:S:AT39 (3132613)	65	2.6 ± 0.6	5.4 ± 1.8
	Treponema sp. I:T:AT24 (3132614)	105	2.6 ± 0.7	5.3 ± 1.8
	Treponema sp. II:10:D12 (2586353)	69	2.5 ± 0.7	5.3 ± 1.8
	Treponema sp. II:D:G93 (2586355)	89	2.5 ± 0.5	5.1 ± 1.7
	Treponema sp. IV:17B:C21 (2586361)	68	2.4 ± 0.7	5.1 ± 1.6
	Treponema sp. IV:17B:C21 (2586361)	57	2.7 ± 0.7	5.7 ± 1.9
	Treponema vincentii (2764819)	291	2.5 ± 0.5	5.1 ± 1.7
Unidentified	Uncultured human oral bacterium A35 (6671223)	104	2.7 ± 0.7	5.7 ± 1.9

2.6. DNA microarray calibration

Probes on the microarrays were calibrated using a dilution series of labeled pooled PCR products of all samples. A two-fold dilution series for the NimbleGen array was created using the following quantities of labeled PCR amplicons: 11.99, 7.72, 3.09, 1.54, 0.77, and 0.39 μg in 12.5 μl. Signal intensities were collected and stored in a database. Two independent calibration procedures were conducted i.e., calibration of individual probes and calibration of probe aggregates. An automatic fitting procedure determined the best fit curve – the calibration curve – (e.g., Langmuir, Linear or Freundlich) as well as the curve parameters from the dilutions and signal intensities. For the individual probe calibration, signal intensity was that of the individual probe. For the probe aggregate calibration, signal intensity was a sum of signal intensities of the probes specific to the corresponding targets. To calculate the abundances of the targets, the calibration curve equations were inverted such that from the signal intensity a dilution factor could be obtained. The dilution factor is proportional to the concentration of the target; hence, it was defined as the abundance of the target.

Details of the calibration protocols to calculate gene abundance are provided in our recent papers (Pozhitkov et al., 2014a; Harrison et al., 2013). The quantities of samples loaded onto the microarray of processing of the individual patients were 3.2 ± 0.25 μg (average of 16 patients ± standard deviation).

2.7. Bioinformatic analyses

Matching the probes to the targets (in silico) and determining the gene abundances using the unique calibrated probes were conducted using custom-designed programs written in C++. *t*-Tests were analyzed and histograms were made using SAS JMP 7 and MS Excel.

3. Results

3.1. Microarray probe coverage and selection

In total, 276,234 probes (25 nt) were synthesized on NimbleGen microarrays. In silico matches of the probes to all 597 oral bacterial sequences (used to design the probes) revealed that 175,206 probes were unique to a single 16S rRNA gene target. To determine if the microarray design had sufficient coverage, we downloaded the curated core oral microbiome 16S rRNA gene sequence dataset from http://microbiome.osu.edu and matched the unique probes to the 1045 microbial sequences (Griffen et al., 2011). We found that 66,716 probes were unique to the 1021 microorganisms in the core oral database. In other words, the unique probes on our microarray had 97.7% coverage of the sequences in the curated core oral microbiome.

We further refined the "concept of unique probes" by removing 3 nucleotides on either end (5′- and 3′-) of the probe (in silico) and matching the shorter probes to the 597 16S rRNA bacterial sequences. The reason for removing these nucleotides was that nucleotides at either end could potentially cross-hybridize to non-specific targets in the microarray experiments (Pozhitkov et al., 2006). In this experiment, probes were considered unique if the 19 nt core matched one 16S rRNA gene target in the dataset (i.e., 597 oral bacterial sequences). Using the 19 nt core, we found that 36,488 of the original 175,206 probes could be susceptible to cross-hybridization, which left 138,718 unique probes that were used in all subsequent analyses to determine gene abundance. Therefore, the entire sequence (25 nt) of the 138,718 unique probes was used in all subsequent experiments.

3.2. Calibration of microarray probes

All probes were calibrated using a dilution series as outlined in our previous studies (Pozhitkov et al., 2014a; Harrison et al., 2013; Hunter et al., 2016a; Pozhitkov et al., 2016a). The Langmuir and Freundlich isotherm equations were fitted to the data and the one with the best fit (R^2) was retained. Examples of two calibrated probes are shown in Fig. 1. Briefly, the fitting algorithm transforms the data to obtain a straight line; two parameters, *a* and *b*, are calculated. Depending on the type of the curve corresponding to the best fit, parameters *a* and *b* are utilized according to the formula of the curve. The parameters of the retained equations and fits are shown in Fig. 1. Apparently, the signal intensities of the dilution data for Probe 62 were best explained by Langmuir and the dilution data for Probe 66 were best explained by Freundlich. For the Freundlich equation,

$$y = e^a x^b,$$

for the Langmuir equation,

$$y = \frac{b^{-1}(b/a)x}{1 + (b/a)x},$$

where *y* is the signal intensity, and *x* is the dilution.

These equations were used to back-calculate the gene target concentration from signal intensity values. In the case of Probe 62 and at a photomultipler tube (PMT) setting of 600, $a = 0.00797$, $b = 0.00034$, x = dilution factor in the calibration series, the signal intensity (*SI*) responds as follows: $SI = 2906.757 * 0.431679 * x / (1 + 0.431679 * x)$. Inverting this equation allows one to calculate the abundance of a gene targeted by this probe. For example, for one of the healthy patients, signal intensity was 1793.89 RFU. This yields a gene abundance of $1793.89 / ((2906.757–1793.89) * 0.431679) = 3.7$ a.u.

In the case of Probe 66 at PMT = 600 where $a = 6.51828$, $b = 0.649462$, x = dilution factor in the calibration series, the *SI* responds according to Freundlich equation as $SI = \exp.(6.51828) * x^{0.649462}$. Inverting this equation allows one to calculate the abundance of a gene targeted by this probe. For example, for one of the healthy patients, SI = 1268.67 RFU. This yields a gene abundance of $(1268.67 / \exp.(6.51828))^{1/0.649462} = 2.6$ a.u.

Probe aggregates were calibrated same way as individual probes. For the probe aggregates, only linear (i.e., $y = ax + b$) and Freundlich curves were evaluated.

3.3. Individually calibrated probes

Three PMT settings (i.e., 500, 600, 700) were used to assess the signal intensities of the hybridized duplexes because we did not know a priori which settings were optimal (i.e., the setting that yielded the minimum signal saturation with maximum dynamic range). The probes at each setting were independently calibrated. In total, 246,232, 246,233, and 207,006 probes were calibrated for the microarrays with the 500, 600, and 700 PMT settings, respectively. In terms of percent of the total probes, about 89% of the probes were calibrated at the 500 and 600 PMT settings and about 75% of the probes at the 700 setting. The lower percent of calibrated probes at the 700 PMT setting was due to saturation of the probes.

Median abundances ± median absolute deviation (MAD) for the 576 rRNA genes and 16 patient samples was assembled into a dataset (Table 2 in reference Hunter et al., 2016b). Note that 21 of the original 597 gene targets were not targeted by the unique probes. The average number of calibrated probes per 16S rRNA gene was 194 ± 214 (avg. ± s.d.). The unique probes not yielding abundances for any of the 16 patients were excluded. Any gene abundance that was >10 a.u. was set to 10 a.u. because we did not expect the calibrations to forecast accurately beyond the dilution series used to create it. Moreover, 10.0 a.u. represents 10 times the amount of DNA hybridized to a microarray based on manufacturer's recommendation. The average gene abundance for the 500, 600, and 700 datasets was 3.5 ± 2.0, 4.1 ± 2.1, and 3.5 ± 2.0 arbitrary units (a.u.), respectively. The lowest and highest gene abundances for all datasets were 0.3 and 10.0 a.u., respectively. The

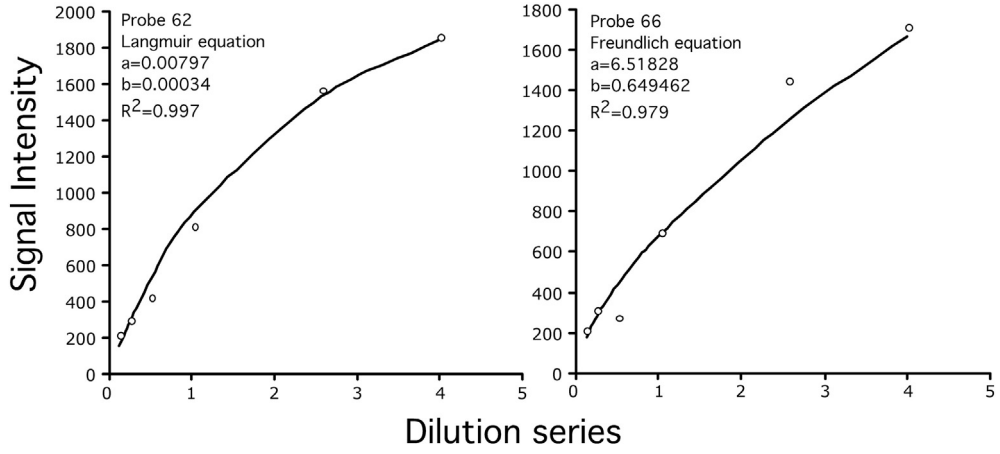

Fig. 1. Calibration curves of two probes. Probe 62 was best calibrated using the Langmuir equation while probe 66 was best calibrated by the Freundlich equation. The equations can be used to back-calculate target concentration (dilution series) from signal intensity.

significance of these findings is that gene abundances could vary by as much as 30-fold and the averaged gene abundances were not significantly affected by PMT settings. Based on these results, all subsequent analyses of the individualized probes were performed using the 600 PMT data. The range of abundances for these data was 0.3 to 10 a.u.

3.4. Examples: selected microbial abundances

Statistical parameters (averages, standard deviations (s.d.), quartiles, and median) for all 576 rRNA genes in 16 patients were calculated and two examples are provided below. The histograms and whisker plots of the *Tannerella forsythia* 16S rRNA gene show the binned distribution of gene abundances in 16 patients based on 100 unique probes (Fig. 2). The gray bars represent the frequencies of the binned abundances and the red bars represent the median abundances. The whisker plots show the mean, standard deviations, median, and quartiles of the abundances. The median abundance (± MAD) in the 4 patients with health was 3.0 ± 0.6 a.u., 3.0 ± 0.2 a.u., 2.2 ± 0.3 a.u., and 1.7 ± 0.3 a.u. (samples HM15, HF8, HF12 and HM10, respectively). The average gene abundance (average ± s.d.) for patients with health was 2.4 ± 0.6 a.u. The abundance of this gene in the 4 patients with edentulism, periodontitis, or caries was: 5.2 ± 1.7 a.u., 4.5 ± 1.4 a.u., and 3.5 ± 2.7 a.u., respectively. Two-tailed *t*-tests revealed significant differences in the average abundances for the 4 patients with edentulous and 4 patients with health ($P < 0.05$), and the 4 patients with periodontitis and 4 patients with health ($P < 0.05$). None of the other paired conditions for this gene was significantly different from one another (Table 1).

The histograms and whisker plots of the *Treponema denticola* 16S rRNA gene show the distribution of abundances in the 16 patients using 94 unique probes (Fig. 3). The average abundances of patients with health, edentulism, periodontitis, and caries were: 2.5 ± 0.6 a.u., 5.3 ± 2.0 a.u., 4.4 ± 1.6 a.u., and 3.8 ± 3.3 a.u., respectively. A two-tailed *t*-test revealed a difference in average abundances for the 4 patients with health and 4 patients with edentulous but only approached significance ($P \leq 0.06$) (Table 2). None of the other paired conditions (i.e., caries versus edentulism, caries versus periodontitis, caries versus health,

Table 2
Bacterial species having significantly different abundances for patients with health versus those with periodontitis based on calibrated individual probes. n_probes, number of probes used to determine the species abundance; Two-sided *t*-tests were based on four patients with health and four patients with periodontitis and alpha = 0.05.

Phylum/class	Genus/species/GI number	*n*_probes	Abundance (X ± s.d.) (a.u.) by	
			Health	Periodontitis
Actinobacteria/Actinobacteria	*Actinomyces odontolyticus* (853707)	36	2.2 ± 0.5	4.1 ± 1.1
	Propionibacterium avidum DSM 4901 (2644976)	53	2.7 ± 0.7	4.7 ± 1.3
Bacteroidetes/Bacteroidetes	*Prevotella nigrescens* ATCC 33563 (294425)	57	2.4 ± 0.6	4.4 ± 1.3
	Tannerella forsythia (289382)	50	2.4 ± 0.6	4.5 ± 1.4
Bacteroidetes/Flavobacteria	*Capnocytophaga sp.* oral clone X089 (9988944)	37	2.6 ± 0.6	4.9 ± 1.4
Firmicutes/Bacilli	*Streptococcus gordonii* (2183315)	14	2.7 ± 0.9	4.7 ± 0.9
	Streptococcus sp. oral clone BW009 (9988906)	11	2.7 ± 0.6	5.1 ± 1.3
	Streptococcus sp. oral strain T1-E5 (14537933)	19	2.6 ± 0.6	4.3 ± 1.1
	Streptococcus sp. oral strain T4-E3 (14537934)	46	2.3 ± 0.5	4.3 ± 1.3
Firmicutes/Clostridia	*Selenomonas* sp. oral clone AA024 (9837490)	96	2.6 ± 0.7	4.7 ± 1.3
Firmicutes/Cocci	*Staphylococcus capitis* (576605)	18	2.5 ± 0.5	4.3 ± 1.1
Fusobacteria	*Fusobacterium periodonticum*	39	2.6 ± 0.7	4.7 ± 1.3
Proteobacteria/Betaproteobacteria	*Neisseria flava* strain U40 (5327199)	1	2.7 ± 0.8	4.8 ± 0.8
	Neisseria sp. oral clone AP015 (10048301)	15	2.7 ± 0.7	5.2 ± 1.4
	Neisseria sp. oral strain B33KA (10048304)	74	2.7 ± 0.7	5.1 ± 1.6
	Neisseria subflava (5327189)	12	2.8 ± 0.9	5.0 ± 1.3
Proteobacteria/Deltaproteobacteria	*Desulfobulbus* sp. oral clone CH031 (10048312)	113	2.6 ± 0.6	4.7 ± 1.4
Proteobacteria/Gammaproteobacteria	*Klebsiella pneumoniae* strain ATCC13884T (3282032)	8	2.3 ± 0.6	4.3 ± 0.9
	Pseudomonas aeruginosa strain LMG 1242T (1907091)	27	2.9 ± 0.8	5.6 ± 1.8
Spirochaetes/Spirochaetes	*Treponema pectinovorum* 8:A:33768 (3757506)	72	2.6 ± 0.6	4.9 ± 1.5
	Treponema pectinovorum OMZ831 (10764812)	108	2.7 ± 0.8	4.9 ± 1.5
	Treponema socranskii (2653630)	35	2.4 ± 0.5	4.3 ± 1.1
	Treponema sp. II:C:T1 (2586354)	57	2.7 ± 0.7	5.1 ± 1.4
Unidentified	Human oral bacterium C20 (6671248)	2	2.3 ± 0.5	5.5 ± 1.5

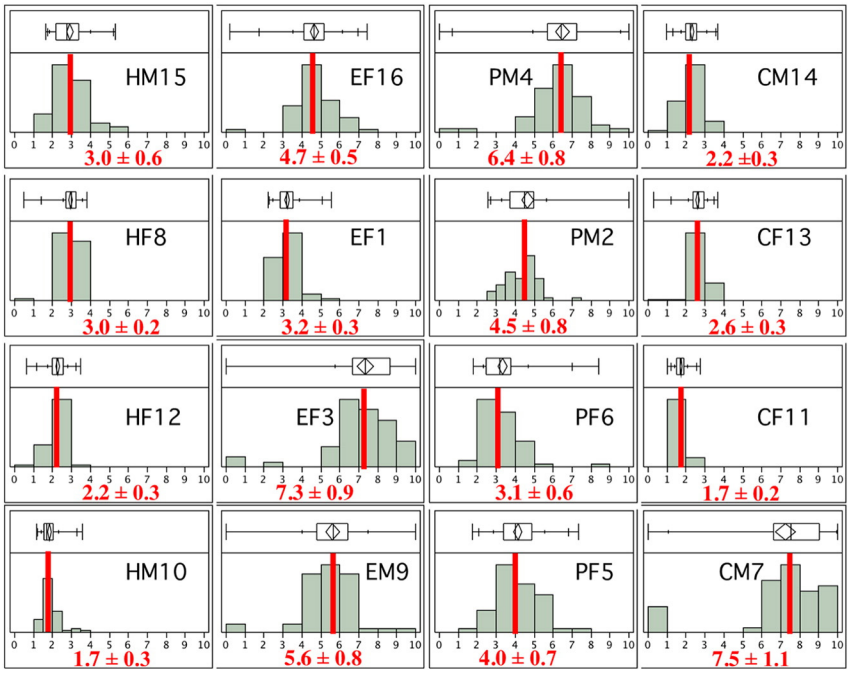

Fig. 2. Distribution of 16S rRNA gene abundances for *Tannerella forsythia* (GI 289382) in 16 patients based on 50 calibrated probes. Each patient has an identifier. The first letter indicates the condition: H, health; E, edentulism; P, periodontitis; C, caries. The second letter indicates patient sex: M, male; F, female. The third number indicates patient number. The red bar indicates median value. Corresponding median absolute deviation (MAD) values are shown. Two-tailed *t*-tests showed that *T. forsythia* was at significantly higher abundance in patients with periodontitis (average ± s.d.; 4.5 ± 1.4 a.u) than those in health (2.4 ± 0.6 a.u) ($P < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

edentulism versus periodontitis, or health versus periodontitis) for this gene yielded differences from one another.

3.5. Microbial abundance signatures

To identify microbial abundance signatures (i.e., 16S rRNA genes) that are unique to a particular condition, we compared the 576 rRNA sequences by condition (caries, edentulism, periodontitis and health) using two-tailed *t*-tests with unequal variance at alpha = 0.05. No significant differences were found for the caries versus edentulism, caries versus health, caries versus periodontitis, or edentulism versus periodontitis conditions. However, specific microbial abundance signatures were found in health versus edentulism and health versus periodontitis conditions (Tables 1 and 2), which are described below.

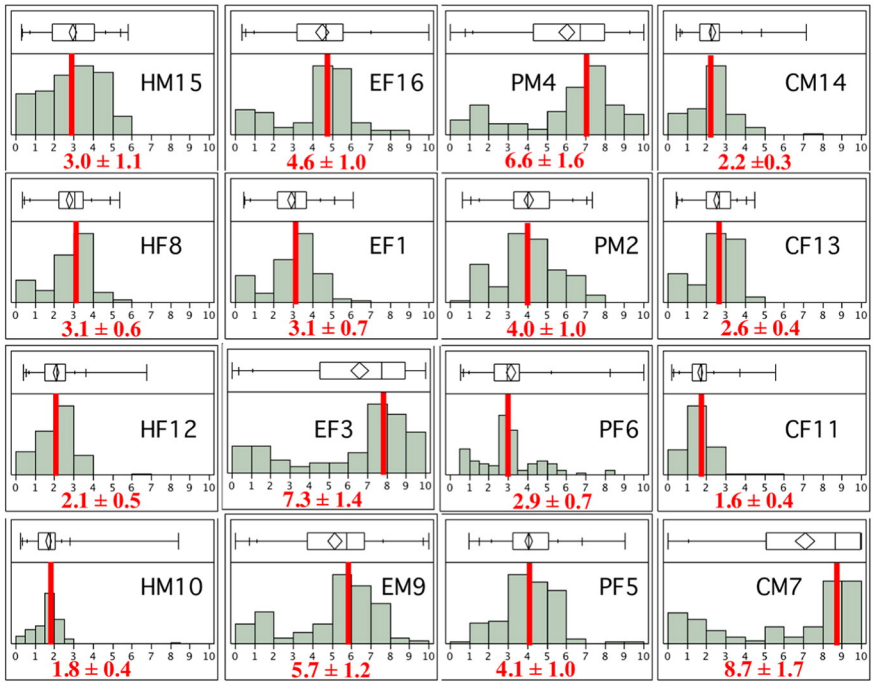

Fig. 3. Distribution of 16S rRNA gene abundances for *Treponema denticola* ATCC 35405 (GI 4580728) in 16 patients based on 94 calibrated probes. Red bar indicates median value. Corresponding MAD values are shown. There is a tendency for *T. denticola* to be more abundant in patients with periodontitis (average ± s.d.; 4.4 ± 1.6 a.u) than those with health (2.5 ± 0.6 a.u) ($P < 0.09$).

3.6. Health versus edentulism

Patients with edentulism had higher abundances of 26 genera and 35 bacterial species than patients associated with dental health. The bacterial species included those in the phyla Actinobacteria, Bacterioidetes, Firmicutes, Fusobacterium, Proteobacteria, and Spirochaetes (Table 1). The number of unique probes used to determine gene abundances of these microorganisms ranged from 6 to 387. Similar to the above, highly related strains (e.g., *Actinomyces odontolyticus* GI 853707 and *A. odontolyticus* GI 69977974) yielded similar abundances even though they were based on different probes (i.e., 36 versus 31 unique probes, respectively). This phenomenon was observed in different strains and species of the genera *Actinomyces*, *Prevotella*, *Capnocytophaga*, *Streptococcus*, *Eubacterium*, *Selenomonas*, *Dialister*, *Leptotrichia*, *Kingella*, *Neisseria*, *Klebsiella*, and *Treponema*.

3.7. Health versus periodontitis

Patients with periodontitis had higher abundances of 14 genera and 21 bacterial species than patients with health. The bacterial genera included those found in the phyla Actinobacteria, Bacterioidetes, Firmicutes, Fusobacterium, Proteobacteria, and Spirochaetes (Table 2). Note that the number of unique probes used to determine gene abundances varied from one to 113. We emphasize this point because additional probes targeting a particular 16S rRNA gene presumably improved the precision. Interestingly, strains of highly related species (e.g., *Streptococcus* sp.; GI 14537933 and GI 14537934) yielded similar abundances even though they were based on different probes (i.e., 19 versus 46 unique probes, respectively). This phenomenon was also observed in different strains and species of the genera *Streptococcus*, *Neisseria* and *Treponema*. This finding provides support for the precision of Gene Meters in terms of gene abundances.

4. Calibrated probe aggregates

The PMT settings affected the number of total genes ($n = 597$) that could be calibrated. Specifically, 564, 567, and 572 genes were calibrated for the PMT settings of 500, 600 and 700, respectively. The abundances for these genes in the 16 patient samples were assembled into a dataset by PMT (Table 3 in reference Hunter et al., 2016b). As before, gene abundances that were greater than 10 a.u. were set to 10 a.u. because we did not expect calibration to forecast accurately beyond the dilution series used to create it. The average gene abundance for the 500, 600, and 700 datasets was 2.9 ± 1.8, 3.4 ± 1.9, and 4.0 ± 2.1 arbitrary units (a.u.), respectively. The lowest and highest gene abundances for all datasets were 0.03 and 10.0 a.u., respectively. Therefore, gene abundances could vary by as much as 300-fold and the averaged gene abundances were affected by PMT settings, with higher abundances at higher PMT settings. Based on these results, all subsequent analyses of the aggregated calibrated probes were performed using the 600 PMT setting data.

4.1. Microbial abundance signatures

Microbial abundance signatures (i.e., 16S rRNA genes) were determined by comparing the 567 rRNA sequences by condition (caries, edentulism, periodontitis and health) using two-tailed *t*-tests with unequal variance at alpha = 0.05. Although significant differences were not found for the caries versus health comparison, specific microbial abundance signatures were found for the caries versus edentulism, caries versus periodontitis, edentulism versus health, edentulism versus periodontitis, and health versus periodontitis comparisons, which are described below.

4.2. Caries versus edentulism

Patients with edentulism had significantly higher abundances of *Lactobacillus* sp. (2.8 ± 1.21 a.u.) than patients with caries (0.6 ± 0.42 a.u.) ($P < 0.03$). Hence, *Lactobacillus* sp. was a putative abundance signature for patients with edentulism.

4.3. Caries versus periodontitis

Patients with periodontitis had significantly higher abundances of *Tannerella forsythia* (1.3 ± 0.58 a.u.) than patients with caries (0.4 ± 0.28 a.u.) ($P < 0.04$). Similarly, patients with periodontitis had significantly higher abundances of *Fusobacterium nucleatum* (1.0 ± 0.28 a.u.) than patients with caries (0.4 ± 0.22 a.u.) ($P < 0.01$). The high abundances of these microorganisms indicate a putative signature for periodontitis as these microorganisms were significantly more abundant in patients with periodontitis versus patients with health (see below).

4.4. Edentulism versus Health

Patients with edentulism HAD significantly higher abundances of 32 genera (and two unidentified species) representing 72 different species/strains than patients associated with health. The microorganisms included the phyla Actinobacteria, Ascomycota, Bacterioidetes, Deinococcus-Thermus, Firmicutes, Fusobacterium, Proteobacteria, Spirochaetes, and Tenericutes (Table 3). The phylum Firmicutes contained the most microorganisms ($n = 29$ species) with the following classes: Bacilli, Clostridia, Erysipelotrichia, and Negativicutes. The class Bacilli consisted of *Bacillus clausii*, and several *Lactobacillus* and *Streptococcus* species. The phylum Actinobacteria contained the second most microorganisms ($n = 15$ species) and included the classes Actinobacteria and Coriobacterila. Within the class Actinobacteria were the following genera: *Actinomyces*, *Bifidobacterium*, *Propionibacterium*, and *Stomatococcus*.

4.5. Edentulism and periodontitis

Patients with periodontitis had significantly higher abundances of seven microbial species/strains than patients with edentulism (Table 4). The phyla for these bacteria included Bacteroidetes, Firmicutes, and Fusobacteria. Notable genera included: *Prevotella*, *Tannerella*, and *Fusobacterium*, which are thought to play roles in periodontitis.

4.6. Health and periodontitis

Patients with periodontitis had higher abundances of 30 genera (five not taxonomically identified) and 62 species than patients with health (Table 5). These bacteria were found in the phyla Actinobacteria, Bacteroidetes, Chloroflexi, Deferribacteres, Firmicutes, Fusobacteria, Proteobacteria, and Spirochaetes. Note that there was a higher abundance of members of the "red complex" (specifically, *Porphyromonas gingivalis*, *Tannerella forsythiai Treponema denticola*) in patients with periodontitis than patients with health.

5. Comparison of calibration methods

Correlation analysis was used to compare calibration methods. While in theory the abundances should be similar by calibration method and the correlations close to one, the abundances determined by aggregated probes were anticipated to be more robust than individual probes when the gene target abundances approached zero. The reason for this is that the sum of all signal intensities obtained from multiple probes targeting the same gene will always be greater than the signal intensities of individual probes. At low gene target abundances, the signal intensities of individually calibrated probes approach the resolution of the scanner, which means the potential for noise in the signal would be minimized in the aggregated probe results.

Table 3
Microbial species having significantly different abundances for patients with health versus those with edentulism using calibrated aggregated probes. n_probes, number of probes used to determine the species abundance; Two-sided T-tests were based on four patients with health and four patients with edentulism and alpha = 0.05.

Phylum/class	Genus/species/GI number	*n*_probes	Abundance (X ± s.d.) (a.u.) by	
			Health	Edentulism
Actinobacteria/Actinobacteria	*Actinomyces gerencseriae* (1838939)	193	1.9 ± 0.36	3.1 ± 0.80
	Actinomyces israelii (1838944)	314	1.6 ± 0.26	2.7 ± 0.67
	Actinomyces meyeri (1838945)	74	2.1 ± 0.41	4.4 ± 1.42
	Actinomyces odontolyticus (6997974)	31	2.5 ± 0.48	5.4 ± 1.73
	Actinomyces odontolyticus (853707)	36	1.6 ± 0.32	2.7 ± 0.54
	Actinomyces sp. (14537925)	310	1.7 ± 0.31	3.0 ± 0.86
	Actinomyces sp. (14537930)	56	2.4 ± 0.52	5.0 ± 1.64
	Actinomyces sp. (14537912)	82	2.0 ± 0.42	4.0 ± 1.26
	Actinomyces sp. (14537929)	168	1.8 ± 0.30	3.5 ± 0.32
	Actinomyces sp. (2073386)	131	1.6 ± 0.21	2.6 ± 0.62
	Actinomyces viscosus (1838951)	98	2.2 ± 0.39	3.7 ± 0.93
	Bifidobacterium sp. (9837452)	205	2.0 ± 0.33	3.4 ± 0.89
	Propionibacterium avidum (2644976)	53	2.5 ± 0.54	5.4 ± 1.82
	Stomatococcus mucilaginosus (2764819)	291	1.8 ± 0.17	2.8 ± 0.34
Actinobacteria/Coriobacteriia	*Atopobium rimae* (10719609)	22	2.7 ± 0.62	7.6 ± 2.13
Ascomycota/*Saccharomycetes*	*Candida albicans* (2507)	1442	2.2 ± 0.46	4.7 ± 1.56
Bacteroidetes/Bacteriodetes	*Porphyromonas-like* sp. (9988935)	631	1.9 ± 0.31	3.1 ± 0.82
	Prevotella sp. (9988921)	87	2.6 ± 0.56	5.6 ± 1.94
	Prevotella sp. (14537919)	88	2.6 ± 0.60	5.7 ± 2.02
	Prevotella sp. (9988918)	127	1.9 ± 0.33	3.2 ± 0.85
	Prevotella sp. (9988919)	98	2.2 ± 0.37	3.7 ± 0.97
Bacteroidetes/Flavobacteria	*Capnocytophaga* sp. (9988943)	101	2.7 ± 0.62	5.9 ± 2.08
Bacteroidetes/Sphingobacteria	*Pedobacter* sp. (14537940)	418	2.2 ± 0.44	4.4 ± 1.39
Deinococcus-thermus/Deinococci	*Deinococcus* sp. (14537950)	790	2.1 ± 0.43	4.2 ± 1.39
Firmicutes/Bacilli	*Bacillus clausii* (9715774)	27	2.2 ± 0.43	4.7 ± 1.57
	Lactobacillus gasseri (175028)	309	2.1 ± 0.45	4.5 ± 1.54
	Lactobacillus paracasei (1808583)	79	1.7 ± 0.32	2.7 ± 0.71
	Lactobacillus sp. (9988912)	73	0.5 ± 0.13	2.8 ± 1.21
	Paenibacillus sp. (15004607)	149	2.0 ± 0.35	3.6 ± 1.04
	Streptococcus gordonii (2183315)	14	1.8 ± 0.33	2.8 ± 0.65
	Streptococcus mitis (9988909)	6	2.7 ± 0.80	5.7 ± 1.95
	Streptococcus pneumoniae (2183314)	29	1.9 ± 0.40	4.1 ± 1.33
	Streptococcus salivarius (176047)	28	1.9 ± 0.40	4.1 ± 1.10
	Streptococcus sp. (9988907)	91	2.5 ± 0.60	5.4 ± 1.87
	Streptococcus sp. (16612185)	83	2.1 ± 0.45	4.3 ± 1.38
	Streptococcus thoraltensis (2578813)	275	2.3 ± 0.49	4.9 ± 1.60
Firmicutes/Clostridia	*Butyrivibrio* sp. (9837461)	569	1.8 ± 0.35	3.5 ± 1.10
	Eubacterium limosum (174517)	437	2.1 ± 0.58	4.5 ± 1.58
	Eubacterium minutum (4001750)	60	1.2 ± 0.21	2.0 ± 0.51
	Eubacterium saphenum (1513214)	447	1.7 ± 0.30	3.0 ± 0.77
	Eubacterium sp. (14537976)	196	2.1 ± 0.37	3.6 ± 0.96
	Eubacterium sp. (14537971)	80	1.5 ± 0.43	2.9 ± 0.87
	Eubacterium sp. (14537961)	219	1.9 ± 0.32	3.4 ± 0.88
	Peptostreptococcus anaerobius (175621)	132	1.7 ± 0.28	2.9 ± 0.78
	Peptostreptococcus asaccharolyticus (454180)	439	1.9 ± 0.42	3.5 ± 0.98
	Eubacterium sp. (3861469)	515	2.0 ± 0.35	3.3 ± 0.87
Firmicutes/Erysipelotrichia	*Erysipelothrix tonsillarum* (7678893)	478	1.8 ± 0.34	3.4 ± 1.03
	Solobacterium sp. (9988916)	113	1.9 ± 0.39	3.5 ± 1.08
Firmicutes/Negativicutes	*Selenomonas flueggei* (9837496)	95	1.7 ± 0.35	3.3 ± 1.05
	Selenomonas flueggei (9837497)	50	2.2 ± 0.53	4.8 ± 1.50
	Uncultured Selenomonas (14161358)	84	2.7 ± 0.70	5.8 ± 2.00
	Uncultured Veillonella (14161350)	47	1.7 ± 0.44	2.9 ± 0.80
	Veillonella dispar (793883)	40	1.8 ± 0.34	3.4 ± 0.81
Fusobacteria/Fusobacteria	*Leptotrichia* sp. (16612191)	400	2.4 ± 0.57	5.0 ± 1.69
	Leptotrichia sp. (14537926)	215	2.4 ± 0.50	4.7 ± 1.48
Proteobacteria/Betaproteobacteria	*Burkholderia* sp. (9988903)	264	2.0 ± 0.35	3.3 ± 0.85
	Kingella denitrificans (174983)	63	2.2 ± 0.36	3.6 ± 0.91
	Neisseria sp. (10048304)	74	2.7 ± 0.57	5.9 ± 2.05
Proteobacteria/Epsilonproteobacteria	*Campylobacter* sp. (10048314)	239	1.7 ± 0.33	3.0 ± 0.84
Proteobacteria/Gammaproteobacteria	*Ewingella americana* (903619)	272	2.0 ± 0.39	4.0 ± 1.30
	Klebsiella pneumoniae (3282032)	8	2.2 ± 0.53	4.8 ± 1.61
Spirochaetes/Spirochaetes	*Treponema denticola* (4580728)	94	1.0 ± 0.16	1.7 ± 0.41
	Treponema pectinovorum (10764812)	108	2.6 ± 0.64	5.6 ± 1.95
	Treponema socranskii (2653628)	77	2.3 ± 0.56	4.7 ± 1.52
	Treponema sp. (3132614)	105	2.7 ± 0.51	4.5 ± 1.50
	Treponema sp. (2586354)	57	2.6 ± 0.65	5.7 ± 1.98
	Treponema sp. (14537906)	128	09 ± 0.16	1.5 ± 0.43
	Treponema sp. (6049684)	85	2.7 ± 0.71	6.0 ± 2.04
	Treponema sp. (2586355)	89	1.4 ± 0.21	2.3 ± 0.56
Tenericutes/Mollicutes	*Mycoplasma buccale* (4883887)	191	2.6 ± 0.61	5.8 ± 1.95
Unknown	Human oral (6671248)	2	2.7 ± 0.60	4.9 ± 1.29
	Uncultured human (6671223)	104	2.6 ± 0.64	5.9 ± 1.91

Table 4
Microbial species having significantly different abundances for patients with edentulism versus those with periodontitis based on aggregated probes. n_probes, number of probes used to determine the species abundance; two-sided *t*-tests were based on four patients with edentulism and four patients with periodontitis and alpha = 0.05.

Phylum/Class	Genus/Species/GI number	*n*_probes	Abundance (X ± s.d.) (a.u.) by	
			Edentulism	Periodontitis
Bacteroidetes/Bacteriodetes	*Prevotella denticola* (294420)	49	1.5 ± 0.36	2.1 ± 0.31
	Prevotella nigrescens (294425)	57	1.0 ± 0.30	1.7 ± 0.39
	Prevotella sp. (9988923)	53	0.8 ± 0.21	1.7 ± 0.45
	Prevotella veroralis (294427)	41	1.2 ± 0.29	2.0 ± 0.42
	Tannerella forsythia (6539450)	94	0.4 ± 0.13	1.3 ± 0.58
Firmicutes/Clostridia	*Mogibacterium vescum* (4140242)	83	0.7 ± 0.55	2.5 ± 1.23
Fusobacteria	*Fusobacterium nucleatum* (4490387)	40	0.5 ± 0.14	1.0 ± 0.28

A plot of the relationship between the correlations of the calibration methods against the average target abundances is shown in Fig. 4. While only 65 out of the possible 567 (~11.5%) microorganisms (GIs) had correlations of less than 0.95, most microorganisms ($n = 502$) (i.e., GIs) had similar abundances (~88.5% had correlation ≥ 0.95). Fig. 4 shows that when the average abundance was less than 2 a.u., the correlations were less than 0.95 (Fig. 4). The significance of this finding is that it provides support that the aggregate probe calibration is more precise than the individual probe calibration.

6. Gene Meter versus DNA sequencing results

In theory, one would expect the same microorganisms to be identified by both approaches (Gene Meters and DNA sequencing) since the source of DNA was the same PCR amplification products from the same 16 patient samples.

For the Gene Meter approach, we detected 576 microbial targets (564 non-redundant targets) (Fig. 5). Thirty-five of the 564 non-redundant targets could not be taxonomically resolved: 13 were classified as "unculturable" and 22 could not be identified to the genus level. Subtracting these 35 ambiguous targets from the 564 non-redundant gene targets left 107 microbial genera.

For the DNA sequencing approach, we detected 654 targets (583 non-redundant targets) (Fig. 5). Nine of the 583 non-redundant targets yielded ambiguous taxonomy (e.g., "anaerobic bacterium", "rumen bacterium") and 17 were plants (e.g., *Nicotiana sylvestris*, *N. tabacum*, *N. tomentosiformism*, "Onion yellows", *Oryza nivara*, *O. sativa*, and *Phalaenopsis aphrodite*, *Citrus sinensis*, *Cucumis sativus*, *Dioscorea elephantipes*, *Draba nemorosa*, *Drimys granadensis*, *Heliathus annuus*, *Calycanthus floridus*, *Agrostis stolonifera*, *Amborella trichopoda*, *Calycanthus floridus*, *Gossypium barbadense*, and *Gossypium barbadense*). Subtracting these 26 ambiguous targets from the 583 non-redundant gene targets left 152 microbial genera.

The union of the two approaches yielded 55 common microbial genera: *Abiotrophia*, *Actinobacillus*, *Actinomyces*, *Aggregatibacter*, *Atopobium*, *Bacillus*, *Bacteroides*, *Bergeyella*, *Bifidobacterium*, *Bradyrhizobium*, *Burkholderia*, *Butyrivibrio*, *Campylobacter*, *Capnocytophaga*, *Clostridium*, *Corynebacterium*, *Cryptobacterium*, *Desulfovibrio*, *Dialister*, *Eikenella*, *Enterococcus*, *Erysipelothrix*, *Eubacterium*, *Fusobacterium*, *Gemella*, *Granulicatella*, *Haemophilus*, *Kingella*, *Lactobacillus*, *Leptotrichia*, *Megasphaera*, *Microbacterium*, *Micrococcus*, *Mitsuokella*, *Moraxella*, *Mycobacterium*, *Mycoplasma*, *Neisseria*, *Paenibacillus*, *Peptococcus*, *Peptostreptococcus*, *Porphyromonas*, *Prevotella*, *Propionibacterium*, *Pseudomonas*, *Rothia*, *Selenomonas*, *Slackia*, *Staphylococcus*, *Streptococcus*, *Tannerella*, *Treponema*, *Variovorax*, *Veillonella*, and *Xanthomonas*.

Fifty-two of the 107 genera (~49%) identified by the Gene Meter approach were not identified by the DNA sequencing approach. The 52 microbial genera included: *Achromobacter*, *Actinobaculum*,*Afipia*, *Agrobacterium*, *Bartonella*, *Bdellovibrio*, *Brevundimonas*, *Bulleidia*, *Candida*, *Cardiobacterium*, *Caryophanon*, *Catonella*, *Centipeda*, *Chlamydia*, *Comamonas*, *Deinococcus*, *Delftia*, *Dermabacter*, *Desulfobulbus*, *Desulfomicrobium*, *Erythromicrobium*, *Eggerthella*, *Enterobacter*, *Escherichia*, *Ewingella*, *Flavobacterium*, *Flexistipes*, *Helicobacter*, *Holophaga*, *Janthinobacterium*, *Johnsonella*, *Klebsiella*, *Lautropia*, *Leptothrix*, *Leuconostoc*, *Methanobrevibacter*, *Mogibacterium*, *Ochrobactrum*, *Olsenella*, *Oribaculum*, *Pedobacter*, *Porphyromonas-like*, *Proteus*, *Rhizobium*, *Simonsiella*, *Solobacterium*, *Sphingomonas*, *Stenotrophomonas*, *Stomatococcus*, *Suttonella*, and *Tropheryma*.

It is important to note that the same genus (and species) could have different GI numbers. In the work below, the GI number was used (rather than the genus name) because there were instances when a genus with a certain GI was detected by both approaches but the same genus with a different GI number was not detected (details below). Altogether, the 52 genera were represented by 76 GI numbers. Since the average gene abundance maxima for these GIs was 8.6 a.u. (close to the maximum of 10 a.u.) in at least one patient, the microorganisms should have been identified in the DNA sequencing results. Putative reasons why they were not include: (i) the processing of the DNA sequencing reads, (ii) inadequate read depth, and (iii) false positive results, which are discussed below.

6.1. Hidden jewels in the unprocessed sequencing reads

To identify microbial species, DNA sequencing reads are subjected to the various filtering procedures (e.g., singleton removal, minimum read lengths, similarities to existing rRNA databases, e.g., 97% similarity, 100 bp min alignment). The filtering can potentially remove 16S rRNA genes of microbial species that are actually present in the sample but not reported in the final taxonomic assignment. To demonstrate this phenomenon, one could match the unique probe sequences of each microbial species (not observed in the final taxonomic assignment) against all unfiltered DNA sequence reads (i.e., before taxonomic assignments are made). If any of the unique probes happen to match the unprocessed DNA sequences for a particular microbial species, one could infer that filtering procedures were responsible for not identifying the microorganism in the final DNA sequencing results. In other words, the rRNA genes of the microbial species were actually present in the unfiltered DNA sequence reads and indeed detected by the Gene Meter approach. However, they are not present in the final filtered DNA sequencing results.

Using custom-designed C++ programs and *Actinomyces* sp. (GI 2073386) as a positive control (since the 16S rRNA gene of this microorganism was found in all patient samples), we matched the unique probes against the DNA sequencing reads of each patient sample. We found that 67 of the 76 GIs (~88%) were present in the DNA sequencing reads. The 45 genera included: *Achromobacter*, *Actinobaculum*, *Agrobacterium*, *Bartonella*, *Bdellovibrio*, *Bulleidia*, *Candida*, *Cardiobacterium*, *Caryophanon*, *Catonella*, *Centipeda*, *Chlamydia*, *Deinococcus*, *Delftia*, *Dermabacter*, *Desulfobulbus*, *Desulfomicrobium*, *Eggerthella*, *Enterobacter*, *Erythromicrobium*, *Ewingella*, *Flavobacterium*, *Flexistipes*, *Helicobacter*, *Holophaga*, *Janthinobacterium*, *Johnsonella*, *Klebsiella*, *Lautropia*, *Leptothrix*, *Leuconostoc*, *Methanobrevibacter*, *Mogibacterium*, *Ochrobactrum*, *Olsenella*, *Oribaculum*, *Pedobacter*, *Porphyromonas-like*, *Proteus*, *Simonsiella*, *Solobacterium*, *Sphingomonas*, *Stomatococcus*, *Suttonella*,and *Tropheryma*. A histogram of the frequencies of the unique probes for each of the 76 GIs revealed that most organisms had 50 or more unique probes

Table 5
Microbial species having significantly different abundances for patients with health versus those with periodontitis based on aggregated probes. n_probes, number of probes used to determine the species abundance; two-sided *t*-tests were based on four patients with health and four patients with periodontitis and alpha = 0.05.

Phylum/class	Genus/species/GI number	*n*_probes	Abundance (X ± s.d.) (a.u.) by	
			Health	Periodontitis ($n = 4$)
Actinobacteria/Actinobacteria	*Actinomyces* sp. (9837444)	31	2.4 ± 0.52	4.6 ± 1.45
	Actinomyces sp. (10946538)	116	1.7 ± 0.28	2.5 ± 0.52
	Actinomyces odontolyticus (853707)	36	1.6 ± 0.32	2.5 ± 0.48
	Bifidobacterium sp. (9837452)	205	2.0 ± 0.33	3.1 ± 0.71
Bacteroidetes/Bacteriodetes	*Bacteroides-like* sp. (9988930)	688	2.0 ± 0.40	3.6 ± 1.01
	Bacteroides-like sp. (9988936)	620	1.8 ± 0.52	3.0 ± 0.58
	Porphyromonas endodontalis (294287)	89	2.2 ± 0.50	4.0 ± 1.12
	Porphyromonas gingivalis (509140)	89	1.1 ± 0.29	1.9 ± 0.40
	Porphyromonas-like sp. (9988934)	647	1.8 ± 0.36	3.1 ± 0.71
	Porphyromonas-like sp. (9988935)	631	1.9 ± 0.31	3.0 ± 0.53
	Prevotella bivia (294429)	282	2.0 ± 0.41	3.4 ± 0.93
	Prevotella buccae (294432)	352	1.7 ± 0.35	3.0 ± 0.74
	Prevotella buccalis (294430)	307	1.6 ± 0.43	3.1 ± 0.93
	Prevotella dentalis (1565276)	371	1.9 ± 0.35	3.2 ± 0.80
	Prevotella denticola (294420)	49	1.0 ± 0.19	2.1 ± 0.31
	Prevotella enoeca (3114912)	399	1.9 ± 0.36	3.3 ± 0.90
	Prevotella intermedia (294422)	227	1.3 ± 0.21	2.1 ± 0.39
	Prevotella nigrescens (294425)	57	0.8 ± 0.29	1.7 ± 0.39
	Prevotella nigrescens (509071)	154	1.1 ± 0.20	2.3 ± 0.42
	Prevotella oralis (294434)	245	1.6 ± 0.32	2.8 ± 0.62
	Prevotella oris (294428)	65	1.1 ± 0.17	1.9 ± 0.29
	Prevotella pallens (2108322)	89	1.0 ± 0.21	2.0 ± 0.33
	Prevotella sp. (9988918)	127	1.9 ± 0.33	3.0 ± 0.65
	Prevotella sp. (14537917)	400	2.0 ± 0.44	3.6 ± 0.95
	Prevotella sp. (29165644)	317	1.9 ± 0.36	3.2 ± 0.77
	Prevotella sp. (9988917)	89	1.4 ± 0.24	2.2 ± 0.46
	Prevotella sp. (9988923)	53	0.8 ± 0.44	1.7 ± 0.45
	Prevotella sp. (14537973)	456	2.0 ± 0.40	3.5 ± 0.83
	Prevotella sp. (14537923)	229	1.5 ± 0.25	2.3 ± 0.39
	Prevotella sp. (14537920)	268	1.2 ± 0.32	1.9 ± 0.13
	Prevotella sp. (9988929)	433	1.5 ± 0.35	2.4 ± 0.37
	Prevotella sp. (14537960)	112	1.2 ± 0.28	2.1 ± 0.33
	Prevotella sp. (14537927)	68	0.8 ± 0.16	1.3 ± 0.10
	Prevotella sp. (9988920)	85	1.2 ± 0.19	2.1 ± 0.15
	Prevotella tannerae (10039600)	167	1.6 ± 0.28	2.3 ± 0.45
	Prevotella veroralis (294427)	41	0.8 ± 0.23	2.0 ± 0.42
	Prevotella zoogleoformans (289379)	276	1.8 ± 0.32	3.0 ± 0.76
	Tannerella forsythia (10946530)	393	1.3 ± 0.43	2.2 ± 0.32
	Tannerella forsythia (289382)	50	2.3 ± 0.16	3.7 ± 0.47
Bacteroidetes/Flavobacteria	*Capnocytophaga haemolytica* (1199611)	383	2.0 ± 0.39	3.4 ± 0.90
	Capnocytophaga sp. (9988944)	37	2.5 ± 0.56	4.5 ± 1.26
	Capnocytophaga sputigena (289579)	172	2.0 ± 0.35	3.0 ± 0.62
Chloroflexi	*TM7 phylum* (14537928)	174	1.8 ± 0.47	3.0 ± 0.46
Deferribacteres/Deferribacteres	*Flexistipes-like* sp. (9858891)	137	2.0 ± 0.40	3.6 ± 0.99
Firmicutes/Bacilli	*Abiotrophia elegans* (2460062)	125	2.0 ± 0.40	3.6 ± 0.76
	Caryophanon sp. (14537944)	40	2.3 ± 0.51	4.1 ± 1.14
	Granulicatella elegans (3123332)	26	2.5 ± 0.57	4.5 ± 1.23
	Lactobacillus sp. (9988912)	73	0.5 ± 0.13	1.0 ± 0.25
	Staphylococcus epidermidis (1199945)	26	2.6 ± 0.67	4.8 ± 1.45
	Staphylococcus xylosus (1199956)	54	1.5 ± 0.25	2.4 ± 0.48
	Streptococcus gordonii (2183315)	14	1.8 ± 0.33	2.6 ± 0.51
	Streptococcus sobrinus (5578902)	258	2.0 ± 0.32	3.0 ± 0.65
	Streptococcus sp. (9988906)	11	2.7 ± 0.65	4.8 ± 1.40
	Streptococcus sp. (16612184)	45	1.9 ± 0.33	3.5 ± 0.32
Firmicutes/Clostridia	*Butyrivibrio* sp. (9837461)	569	1.8 ± 0.35	3.1 ± 0.74
	Clostridium tetanomorphum (535115)	525	1.8 ± 0.50	3.1 ± 0.81
	Eubacterium infirmum (3299817)	85	1.7 ± 0.36	2.7 ± 0.66
	Eubacterium saphenum (1513214)	447	1.7 ± 0.30	2.8 ± 0.62
	Eubacterium sp. (9837459)	637	2.0 ± 0.42	3.4 ± 0.94
	Eubacterium sp. (14537976)	196	2.1 ± 0.37	3.3 ± 0.70
	Eubacterium sp. (14537903)	39	1.4 ± 0.25	2.0 ± 0.34
	Eubacterium sp. (14537961)	219	1.9 ± 0.32	3.3 ± 0.73
	Eubacterium sp. (14537982)	367	1.3 ± 0.42	2.5 ± 0.50
	Eubacterium sp. (14537971)	80	1.5 ± 0.43	3.1 ± 0.48
	Mogibacterium diversum (6942242)	114	1.6 ± 0.30	2.8 ± 0.72
	Peptostreptococcus anaerobius (175621)	132	1.7 ± 0.28	2.7 ± 0.59
	Peptostreptococcus asaccharolyticus (454180)	439	1.9 ± 0.42	3.1 ± 0.76
	Peptostreptococcus magnus (454441)	401	1.4 ± 0.38	2.1 ± 0.37
	Peptostreptococcus sp. (9837456)	294	1.8 ± 0.36	3.1 ± 0.83
	Eubacterium sp. (3861469)	515	2.0 ± 0.35	3.0 ± 0.68
Firmicutes/Negativicutes	*Dialister* sp. (9837481)	63	2.3 ± 0.56	4.0 ± 1.05
	Dialister sp. (9837479)	330	1.3 ± 0.35	2.0 ± 0.32
	Firmicutes sp. (9837472)	204	1.6 ± 0.34	2.4 ± 0.52

Table 5 (*continued*)

Phylum/class	Genus/species/GI number	*n*_probes	Abundance (X ± s.d.) (a.u.) by	
			Health	Periodontitis ($n = 4$)
	Megasphaera sp. (14537975)	305	2.2 ± 0.49	4.0 ± 1.14
	Megasphaera sp. (9837476)	276	1.8 ± 0.39	3.0 ± 0.75
	Selenomonas sp. (9837490)	96	2.5 ± 0.62	4.7 ± 1.42
	Selenomonas sp. (9837488)	540	2.2 ± 0.47	3.8 ± 1.07
	Veillonella dispar (793883)	40	1.8 ± 0.34	2.7 ± 0.53
Fusobacteria	*Filifactor alocis* (4127826)	538	1.4 ± 0.28	2.8 ± 0.62
	Fusobacterium nucleatum (43402)	15	2.4 ± 0.61	4.2 ± 1.16
Fusobacteria/Fusobacteria	*Leptotrichia* sp. (9837509)	240	2.2 ± 0.52	4.0 ± 1.15
	Leptotrichia sp. (9837506)	172	2.2 ± 0.41	3.8 ± 1.05
	Leptotrichia sp. (9837507)	286	2.2 ± 0.45	3.9 ± 1.02
	Leptotrichia sp. (14537981)	326	1.9 ± 0.42	3.1 ± 0.73
Proteobacteria/Betaproteobacteria	*Neisseria flava* (5327199)	1	2.7 ± 0.76	4.8 ± 0.80
	Neisseria pharyngis (5327180)	12	2.7 ± 0.74	5.0 ± 1.37
	Neisseria sp. (10048301)	15	2.6 ± 0.60	5.1 ± 1.37
Proteobacteria/Epsilonproteobacteria	*Campylobacter* sp. (10048314)	239	1.7 ± 0.33	2.7 ± 0.61
Proteobacteria/Gammaproteobacteria	*Haemophilus segnis* (174775)	124	2.5 ± 0.53	4.5 ± 1.29
	Klebsiella pneumoniae (3282032)	8	2.2 ± 0.53	4.2 ± 1.22
Spirochaetes/Spirochaetes	*Treponema denticola* (4580728)	94	1.0 ± 0.16	2.2 ± 0.61
	Treponema socranskii (2653630)	35	2.2 ± 0.45	4.0 ± 1.17
	Treponema socranskii (2653627)	47	2.6 ± 0.67	4.8 ± 1.39
	Treponema sp. (2586367)	154	2.5 ± 0.61	4.6 ± 1.35
	Treponema sp. (3132614)	105	2.2 ± 0.51	4.0 ± 1.15
	Treponema sp. (2586364)	77	2.5 ± 0.58	4.6 ± 1.28
	Treponema sp. (2586354)	57	2.6 ± 0.65	4.9 ± 1.45
	Treponema sp. (2586361)	68	1.9 ± 0.40	3.3 ± 0.84
	Treponema sp. (14537906)	128	0.9 ± 0.16	2.3 ± 0.68
	Treponema sp. (2586355)	89	1.4 ± 0.21	2.5 ± 0.44
Unknown	Human oral (6671248)	2	2.7 ± 0.60	4.5 ± 0.96
	Uncultured bacterium (9858892)	252	2.1 ± 0.38	3.1 ± 0.61
	Uncultured bacterium (4530523)	98	1.8 ± 0.30	2.8 ± 0.55
	Uncultured bacterium (4680614)	719	1.4 ± 0.42	2.6 ± 0.45
	Uncultured human (6671255)	14	2.8 ± 0.78	5.0 ± 1.45

matching to the DNA sequencing reads, with some having up to 400 matching probes (Fig. 6).

The significance of this result is that many microbial species – not taxonomically identified in DNA sequencing results – were actually present in the patient samples. Moreover, the inclusion of these results into the union of the two approaches increased the number of common microbial genera from 55 to 100 (Fig. 4). In other words, 100 of the 107 genera (~93%) identified by the Gene Meter approach were found in the processed and unprocessed DNA sequencing reads. These results indicate false negatives in the taxonomic assignment of the DNA sequencing results due to filtering and false positives in the Gene Meter results (since 7 were not identified in the sequencing reads).

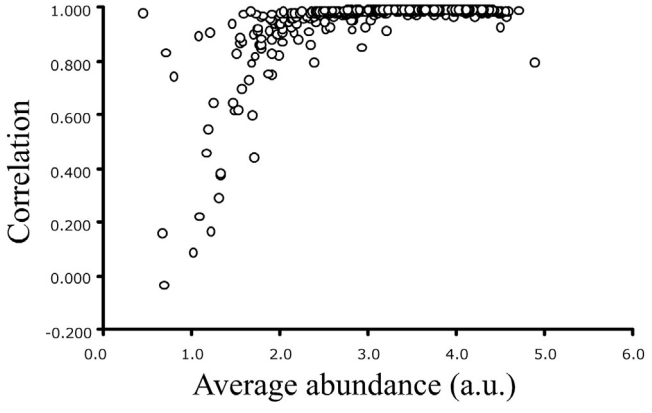

Fig. 4. Correlation of abundances of GIs for two calibration approaches (individual probe calibration and aggregate probes targeting same gene) versus average abundance of the GIs determined using the aggregate calibration approach. Results show the average abundances for GIs are low (<2.0 a.u.). The correlations are also low, presumably because the signal intensities of the individual probes approach the resolution of the scanner while the sum of the signal intensities of aggregated probes do not.

6.2. Microorganisms identified by the Gene Meter approach but not found in the unfiltered sequencing reads

Nine of the 76 GIs (~12%) not detected in any of the unfiltered DNA sequencing reads included: *Afipia* sp. ($n = 191$ probes), *Brevundimonas diminuta* ($n = 224$ probes), *Caryophanon* sp. ($n = 53$ probes), *Comamonas* sp. ($n = 19$ probes), *Delftia* sp. ($n = 4$ probes), *Escherichia coli* ($n = 17$ probes), *Rhizobium loti* ($n = 335$ probes), *Simonsiella muelleri* ($n = 31$ probes), and *Stenotrophomonas maltophilia* ($n = 68$ probes). The number of unique probes is shown because a microbial species with low number might not be detected in the unfiltered reads simply because of the low probability of finding a match. It should be noted that one species of *Leptothrix* (GI 14537943) with $n = 38$ probes did not match any unfiltered sequence reads — however, another species of *Leptothrix* (GI 14537937) with $n = 361$ probes did match 96 sequencing reads. These findings indicate that the number of unique probes did influence whether or not a particular species was found in the unfiltered sequencing reads.

In our study, microorganisms with a low number of unique probes ($n = 38$ or less) included: *Comamonas* sp., *Delftia* sp., *Simonsiella muelleri*, and *Escherichia coli*. We concluded that these microorganisms were probably not detected in the unfiltered sequencing reads because they had too few probes.

The remaining 5 species, detected by the Gene Meter approach but not detected in the unfiltered sequencing reads, had a high number of unique probes (i.e., *Afipia* sp., *Brevundimonas diminuta*, *Caryophanon* sp., *Rhizobium loti*, and *Stenotrophomonas maltophilia*). In theory, these microorganisms should have been detected in the unfiltered sequencing reads — but they were not. There are two possible reasons for this phenomenon. First, there might not have been sufficient read depth of

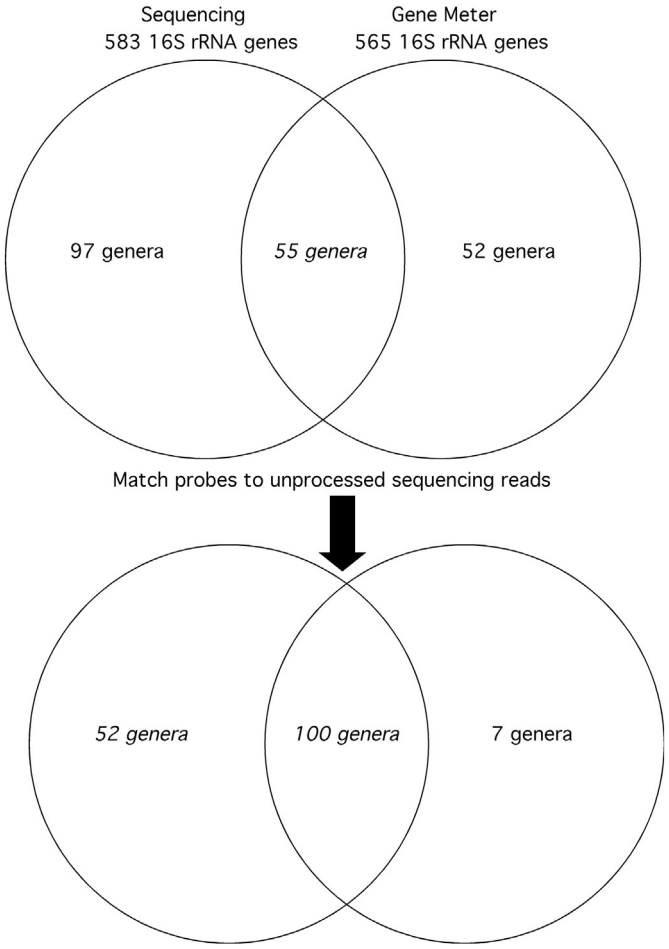

Fig. 5. Shared genera by DNA sequencing and Gene Meter for 16 patients. Top shows taxonomic classification based on processed DNA sequencing reads. Bottom shows taxonomic classification based on probe matches of unprocessed DNA sequencing reads and processed DNA sequencing reads.

the unfiltered sequencing data. According to our previous paper (Table S1 in Ref. Pozhitkov et al., 2016a), the read depth varied by patient sample with the lowest being 2064 reads for a healthy patient and the highest being 23,904 reads for a caries patient. *Afipia* sp. for example,

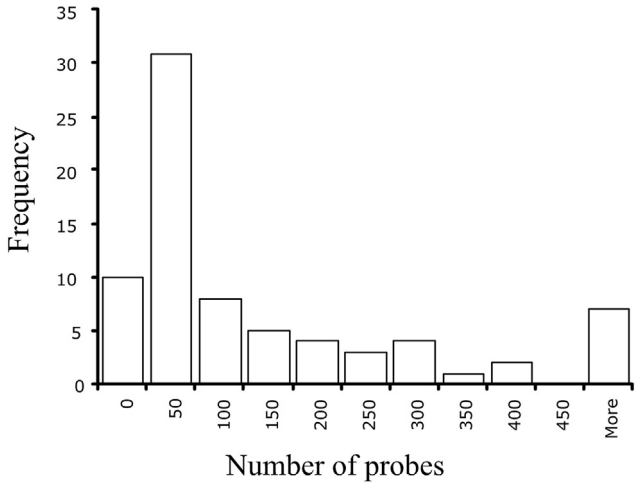

Fig. 6. Number of probes of 76 microorganisms (GIs) belonging to the 52 genera (Fig. 4) matching unprocessed DNA sequencing reads. Most microorganisms had 50 or more probe matches to sequencing reads, indicating they were in fact present in the patient samples (even though they were not detected by MG-RAST presumably due to filtering).

had high abundance in one of the caries patients (8.4 a.u.) and one of the patients with edentulism (7.6 a.u.) (determined by the Gene Meter approach) — yet, the read counts for these samples were 11,532 and 18,515, respectively, indicating that this microorganism should have been identified in the unfiltered DNA sequencing reads. Hence, there is weak support for the argument since the highest abundances (by the Gene Meter approach) occurred in samples that also had moderate to high read counts.

Second, these microorganisms might not have been found in the unfiltered sequencing reads because they are false positives of the Gene Meter approach due to non-specific hybridization to other gene targets. We tested for the potential of non-specific hybridizations by removing the first three nucleotides on the 5′- and 3′-ends from the unique probes (because they might hybridize to non-specific DNA targets) and matching the short probes (19 nt) against the unprocessed sequencing reads. We found that several probes of *Caryophanon* sp., *and Rhizobium loti* and *Stenotrophomonas maltophilia* matched the unfiltered sequencing reads. Specifically, two unique probes (1015327 and 1015321) for *Caryophanon* sp. matched unfiltered sequence reads in a patient with caries and another patient with health. These probes have the potential to non-specifically bind to 16S rRNA sequences of other species in the patient samples and therefore they are false positives.

Ninety-six of the 151 genera (~64%) identified by DNA sequencing were not detected by the Gene Meter approach included: *Acetitomaculum*, *Acetivibrio*, *Acetobacterium*, *Acidithiobacillus*, *Acidovorax*, *Actinoplanes*, *Aerococcus*, *Agrococcus*, *Alistipes*, *Aminobacterium*, *Amycolatopsis*, *Anaerostipes*, *Aquitalea*, *Arcanobacterium*, *Arthrobacter*, *Bavariicoccus*, *Bergeriella*, *Blautia*, *Brachymonas*, *Brenneria*, *Brevibacterium*, *Butyricimonas*, *Carnobacterium*, *Cellulophaga*, *Cellulosimicrobium*, *Chelonobacter*, *Chitinophaga*, *Chryseobacterium*, *Collinsella*, *Cryobacterium*, *Cytophaga*, *Dechloromonas*, *Desulfonispora*, *Desulfosporosinus*, *Desulfotomaculum*, *Enterorhabdus*, *Exiguobacterium*, *Finegoldia*, *Gallibacterium*, *Gardnerella*, *Geobacillus*, *Globicatella*, *Gordonibacter*, *Hespellia*, *Jonesia*, *Kocuria*, *Kytococcus*, *Lactococcus*, *Mannheimia*, *Megamonas*, *Melissococcus*, *Mobiluncus*, *Myroides*, *Odoribacter*, *Pantoea*, *Parabacteroides*, *Paraprevotella*, *Pasteurella*, *Pectinatus*, *Phascolarctobacterium*, *Promicromonospora*, *Pseudobutyrivibrio*, *Pseudonocardia*, *Pyramidobacter*, *Ralstonia*, *Renibacterium*, *Rhodococcus*, *Riemerella*, *Rikenella*, *Robinsoniella*, *Roseburia*, *Ruminococcus*, *Saccharopolyspora*, *Sanguibacter*, *Sebaldella*, *Serratia*, *Spirochaeta*, *Sporomusa*, *Streptobacillus*, *Streptomyces*, *Syntrophococcus*, *Tenacibaculum*, *Terrabacter*, *Tetragenococcus*, *Thermoanaerobacter*, *Thermomonospora*, *Thermus*, *Thiobacillus*, *Tissierella*, *Trichococcus*, *Vagococcus*, *Weeksella*, *Weissella*, and *Xylanimicrobium*. These microorganisms were not detected because the microarray probes did not target their 16S rRNA genes.

6.3. An attempt to calibrate DNA sequencing data

We investigated the feasibility of calibrating the 454 sequencing reads by diluting pooled patient samples with DNA from salmon sperm. We chose salmon sperm because the sequences are dissimilar to microbial 16S rRNA genes and can easily be distinguished. In theory, DNA sequencing reads should yield similar linear equations for the dilution series but, in reality, amplification and sequencing biases vary for different targets and one would expect the slopes of the dilution series to be dissimilar (Fig. 7). Our results show that the slope of *Neisseria* sp. was 69.0, while that of *Porphyromonas gingivalis* was 2818.2. Similarly, the slope of *Eubacterium brachy* was 102.7, while that of *Veillonella* sp. was 397.3. The importance of these findings are two-fold. First, it provides proof of the extreme biases of sequencing reads generated using NGS. Second, the results suggest that it might be possible to obtain precise abundances of 16S rRNA genes using NGS by calibrating 16S rRNA gene reads, before determining gene abundances, similar to what was done with the DNA microarray output using the Gene Meter approach. To our knowledge, this is the first study to provide preliminary evidence

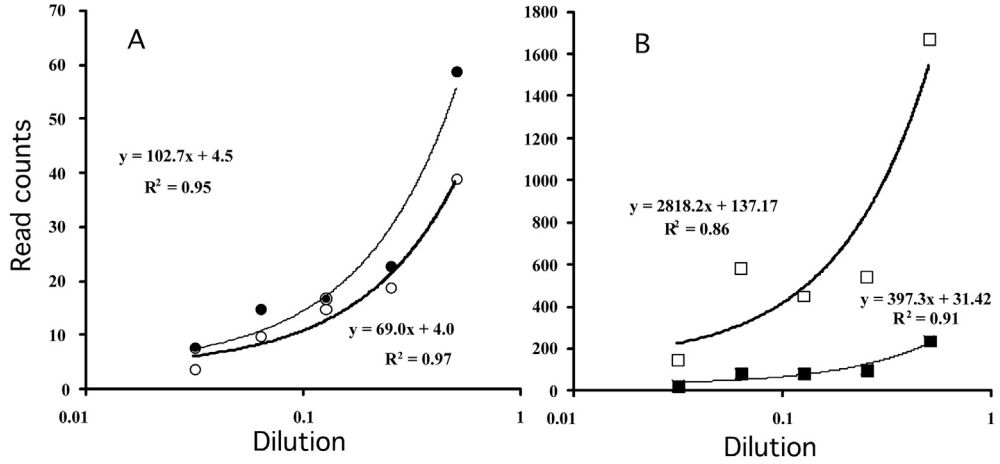

Fig. 7. Calibration of 454 reads using the Gene Meter approach. Panel A: *Neisseria* sp., open circles; *Eubacterium brachy*, closed circles. Panel B: *Porphyromonas gingivalis*, open squares; *Veillonella* sp., closed squares.

that gene abundances might be precisely determined by next-generation sequencing data.

7. Discussion

7.1. DNA microarrays — too early to abandon

A common perception among researchers is that high-throughput DNA sequencing is the best approach to characterize microbial communities because other approaches, such as DNA microarrays are noisy. Indeed, conventional DNA microarrays are noisy (Tu et al., 2002) because the signal is not calibrated and the output has to be normalized for sample comparison. The Gene Meter approach solves the noise problem by calibrating all probes on a microarray before testing biological samples. The calibration identifies probes with noisy behavior because they will not fit the adsorption models. Noisy probes are not used in subsequent analyses. The Gene Meter approach also does not require any normalization of the output because the same amount of DNA is loaded onto every microarray. In other words, the abundances of different samples can be directly compared without introducing biases associated with other methods such as DNA sequencing and conventional DNA microarrays. It is for these reasons that we believe that the Gene Meter approach is superior to existing approaches — particularly when one desires to determine the abundance of a gene in a pool of genes.

It should be noted that DNA sequencing approaches (such as the one used in our previous study; Pozhitkov et al., 2015) have problems too, that limit their ability to determine gene abundance. For example, an extra PCR amplification step is required in DNA sequencing (e.g., emulsion PCR) and that adds biases to the interpretation of the output. The Gene Meter approach does not need any extra amplification step — the labeled DNA is added directly to the microarray. Another problem in DNA sequencing (as shown in this study) is the post-processing of the reads sometimes filter out 16S rRNA genes that are actually present in biological samples (specifically, 40 out of 107 genera in this study). The fact that many of the unique probes of the 40 unidentified genera matched the unprocessed sequencing reads suggest that the taxonomic assignments in other sequencing studies might have grossly underestimated the actual diversity of microorganisms in biological samples.

We recognize that quantitative PCR (qPCR) could also have been used to determine the abundance of 16S rRNA genes in our study. However, like DNA sequencing, this approach is subject to PCR amplification biases and normalization steps. In addition, qPCR can only investigate a limited number of gene targets at one time (i.e., it is not high throughput), while the Gene Meter approach is high throughput but limited to the number of probes on microarray surface.

Similar to all approaches, the Gene Meter approach is not without its problems. For instance, seven genera identified by the Gene Meter approach were not detected in either the processed or unprocessed DNA sequencing reads. Three of the seven genera were classified as false positives as they had the potential to cross-hybridize to the 16S rRNA genes of other species. Although there are several additional explanations for these phenomena (e.g., insufficient DNA sequencing depth; need for better probe design), the Gene Meter approach still provides precise target abundances for many genera and is superior to other approaches because normalization is not required.

A simple solution to prevent normalization biases and improve upon abundance determinations for DNA sequencing is to calibrate the instrument before analyzing samples. This study was the first to demonstrate that a DNA sequencing instrument can be calibrated using a dilution series (Fig. 7). Future studies are now warranted to demonstrate the utility of calibration for DNA sequencing instruments using biological samples.

7.2. Calibration by aggregate probes works optimally

In early Gene Meter studies (Pozhitkov et al., 2014a; Harrison et al., 2013), the dilution series were calibrated using the averaged signal intensities of all replicated probes specific to a gene target. The averaging of the signal reduced noise by minimizing the effects of outliers. Since we did not use identically replicated probes in the present study, we calibrated the DNA microarray two ways using individual probes or aggregated probes. We sought to determine which of the two calibrations was optimal.

Calibration of the individual probes involved modeling the signal intensities of an individual probe in a dilution series. The abundance of the gene in a biological sample was determined by back-calculating the abundances of all probes which target that specific gene using the calibration and then averaging (or determining the median) the gene abundances. For example, consider three calibrated probes targeting the same gene. The abundance of a specific gene by calibrated probe A was 1 a.u., probe B was 2 a.u. and probe C was 3 a.u. Therefore, the final abundance of the gene was the average or median, 2 a.u.

Calibration of the aggregated probes involved summing the signal intensities of all probes in a dilution series that target a specific gene and determining the model that best fits the experimental data. For example, if the signal intensities of probes A, B, and C for a specific gene were 300, 500, and 700 relative fluorescence units (RFU) at one dilution, then the signal intensity used to calibrate the gene would be 1500 RFU. The abundance of a gene in a biological sample was determined by summing the signal intensities of all probes that target the gene and back-calculating the relative abundance from the calibration.

What distinguishes the two calibration approaches is that the aggregate probe approach uses the sum of the signal intensities of all probes as input to the calibration model while the individual probe approach uses only the signal intensity of an individual probe as input to the calibration model. Also, in the aggregate probe approach, the abundance is directly determined from the model, while in the individual probe approach, the final gene abundance is determined by the averaged (or median) abundance of all probes targeting that gene.

We showed that calibrations of aggregated probes were better than individual probes when the gene target abundances were low. The reason for this phenomenon is that the low abundance genes are more affected by signal noise because some readings were close to the level of resolution of the microarray scanner. The sum of the signal intensities of all probes targeting a specific gene minimizes this problem because the noise is not averaged. Our results showed that if the gene target abundance was greater than 2 a.u. (Fig. 4), there were no significant differences in the gene abundances by the calibration approach.

Interestingly, we found more differences in gene abundances by patient condition using aggregated probes (Tables 3, 4 and 5) than individual probes (Tables 1 and 2). This finding suggests that several microorganisms do have differences in abundances by condition — but they occur at low abundances (<2 a.u.). In other words, the differences by condition could only be detected using the aggregated probe approach. Therefore, the aggregated probes was used for examining microbial species differences by condition (below).

7.3. Abundance signatures for dysbiosis

The following 17 genera had significantly higher abundances in patients with periodontitis and patients with edentulism when contrasted with patients with health: *Actinomyces*, *Bifidobacterium*, *Butyrivibrio*, *Campylobacter*, *Capnocytophaga*, *Eubacterium*, *Klebsiella*, *Lactobacillus*, *Leptotrichia*, *Neisseria*, *Peptostreptococcus*, *Porphyromonas*, *Prevotella*, *Selenomonas*, *Streptococcus*, *Treponema*, and *Veillonella*. At the species level this included: *Actinomyces odontolyticus*, *Klebsiella pneumoniae*, *Peptostreptococcus anaerobius*, *Streptococcus gordonii*, *Treponema socranskii*, and *Veillonella dispar*. We classified these microorganisms as 'signatures of dysbiosis' in the human oral microbiome because they occurred in more than one condition. They were excluded from consideration to a single condition below (e.g., a signature for periodontitis).

These microorganisms have previously been reported to be involved in dysbiosis in human oral studies. For example, *Actinomyces* sp. are found in edentulous patients (Danser et al., 1997). *Bifidobacterium* are found in the mouths of edentulous patient wearing dentures (Mantzourani et al., 2010) and associated with root decay and periodontal disease (Dina et al., 2013). *Butyrivibrio*, *Campylobacter*, *Eubacterium*, *Prevotella*, *Selenomonas*, *Streptococcus*, *Leptotrichia*, and *Treponema* are associated with peri-implant communities (Kumar et al., 2012). *Eubacterium*, *Prevotella* and *Lactobacillus* are major genera found in edentulous patients (Könönen et al., 1991). *Prevotella*, *Fusobacterium*, *Leptotrichia*, *Streptococcus*, *Neisseria*, and *Veillonella* were found in edentulous infants (Cephas et al., 2011; Teles et al., 2012; Könönen et al., 1992). *Peptostreptococcus* has been associated with periodontal pockets of partially edentate patients (van Winkelhoff et al., 2002) and is associated with soft tissue inflammation in edentate patients (Danser et al., 1997). *Prevotella* and *Campylobacter* have been observed in edentate patients (Danser et al., 1997). *Treponema Campylobacter*, *Peptostreptococcus*, and *Porphyromonas* are associated with periodontitis (Piovano, 1999; van Winkelhoff et al., 2000). *Veillonella*, *Streptococcus* and *Candida* are often found in the saliva of edentulous patients (Sato et al., 1993).

At the species level, *Actinomyces odontolyticus* has been found in failed dental implants (Sakanaka et al., 2015). *Klebsiella pneumoniae* has been frequently observed in patients with diseased implants (Sarkonen et al., 2005) and patients with periodontitis (Ardila et al., 2010; Gonçalves et al., 2007). *Peptostreptococcus anaerobius* has been found inside root canals (Piovano, 1999). *Streptococcus gordonii* has been shown to be an important player in the development of periodontitis as it is involved in co-adhesion and metabolic interactions with other pathogens (Sakanaka et al., 2015; Hajishengallis and Lamont, 2016). *Treponema socranskii* has been associated with peri-implant diseases (Máximo et al., 2009; Persson and Renvert, 2014), lesions of teeth with apical periodontitis (Rosa et al., 2015), infected root canals and abscesses (Montagner et al., 2010). *Veillonella dispar* is an earlier biofilm colonizer (Mashima and Nakazawa, 2014) and involved in periodontal disease (Moon et al., 2015).

7.4. Abundance signatures for periodontitis

The following 13 genera had significantly higher abundances in patients with periodontitis than those with health: Abiotrophia, Bacteroides-like, *Caryophanon*, *Clostridium*, *Dialister*, *Filifactor*, *Fusobacterium*, *Granulicatella*, *Haemophilus*, *Megasphaera*, *Mogibacterium*, *Staphylococcus*, and *Tannerella*. At the species level this included: *A. elegans*, *C. tetanomorphum*, *F. alocis*, *F. nucleatum*, *G. elegans*, *H. segnis*, *M. diversum*, *S. epidermidis*, *S. xylosus*, and *T. forsythia*. Note that some genera could not be resolved to the species level. These microorganisms were classified as microbial signatures of periodontitis in the oral microbiome.

The following microorganisms have been previously shown to be associated with periodontal disease: *Abiotrophia elegans* (Rego et al., 2010; Mikkelsen et al., 2000), Bacteroides-like (Olsvik et al., 1996), *Dialister* (Lee et al., 2016; Gonçalves et al., 2016; Collins et al., 2016), *Filifactor alocis* (Colombo et al., 2009; Oliveira et al., 2016; Basic and Dahlén, 2015; Aruni et al., 2015; Chen et al., 2015), *Fusobacterium nucleatum* (Topcuoglu and Kulekci, 2015; Danser et al. 1997; Ahn et al., 2016; Mendes et al., 2016; Nickles et al., 2016; Bui, 2016), *Granulicatella elegans* (Lourenço et al., 2014), *Haemophilus* (Colombo et al., 2009; Park et al., 2015; Takeshita et al., 2014), *Haemophilus segnis* (Petsios et al., 1995; Kamma et al., 1995) *Megasphaera* (Kumar et al., 2003; Kumar et al., 2005), *Mogibacterium* (Marchesan et al., 2015; Camelo-Castillo et al., 2015), *Staphylococcus epidermidis* (Fujii et al., 2009), *Tannerella forsythia* (Oliveira et al., 2016; Nickles et al., 2016; Wong et al., 2016; Lanza et al., 2016). These findings support our findings that these microorganisms are signatures of periodontitis in the human oral microbiome.

Although the genera *Clostridium*, *Mogibacterium* and *Staphylococcus* have been associated with periodontal disease (Griffen et al., 2012; VieiraColombo et al., 2016), there is no evidence in the literature suggesting that *Clostridium tetanomorphum*, *Mogibacterium diversum*, *Staphylococcus xylosus* or the genus *Caryophanon* has been associated with periodontitis. Of note, *Mogibacterium diversum* has been associated with caries (Nakazawa et al., 2002) and the genera *Caryophanon* has been found in the gingival flora of dogs (Nyby et al., 1977). Recall that our earlier results suggest that *Caryophanon* might be a false positive because it was not found in the sequencing reads and some probes were shown to have the potential to hybridize with other species.

A probable reason that these microorganisms were not recognized in the literature is because they occurred at low abundances and might be overlooked using alternative technologies (e.g., NGS). For example, of the 525 *Clostridium tetanomorphum* probes, the abundance in patients with health was 1.8 ± 0.50 a.u. versus 3.1 ± 0.81 a.u. in patients with periodontitis. Similarly, of the 114 *Mogibacterium diversum* probes, the abundance in patients with health was 1.6 ± 0.30 a.u. versus 2.8 ± 0.72 a.u. in patients with periodontitis, and of the 54 *Staphylococcus xylosus* probes, the abundance in patients with health was 1.5 ± 0.25 a.u. versus 2.4 ± 0.48 a.u. in patients with periodontitis (Table 5). All comparisons are statistically significant and all species have been previously found in human oral cavities.

7.5. Abundance signatures for edentulism

The following 14 genera had significantly higher abundances in patients with edentulism than patients with health: *Atopobium*, *Bacillus*, *Burkholderia*, *Candida*, *Deinococcus*, *Erysipelothrix*, *Ewingella*, *Kingella*, *Mycoplasma*, *Paenibacillus*, *Pedobacter*, *Propionibacterium*, *Solobacterium*, and *Stomatococcus*. At the species level, this included: *Atopobium rimae*, *Bacillus clausii*, *Candida albicans*, *Erysipelothrix tonsillarum*, *Ewingella americana*, *Kingella denitrificans*, *Mycoplasma buccale*, *Propionibacterium avidum*, and *Stomatococcus mucilaginosus*. While high abundances of *Atopobium* species, *Candida albicans* and *Bacillus* sp. have been previously reported in edentulous patients (O'Donnell et al., 2015), we could not find any evidence for the high abundance of the other genera and species in patients with edentulism in the literature. Hence, this is the first study to show higher abundance of these microorganisms in patients with edentulism.

7.6. Technology rules our understanding of the oral microbiome

Much of what is known about the oral microbiome is based on the technology available at the time of publication. For example, the idea behind the 'red complex' originates from the checkerboard DNA–DNA hybridization technique (Socransky et al., 1994), which was used to examine the microbial composition of plaque in patients in health and periodontitis (Ximenez-Fyvie et al., 2000a; Ximenez-Fyvie et al., 2000b), the salivary microbiota levels in relation to periodontal status (Darout et al., 2002), the relationship of cigarette smoking to the composition of the subgingival microbiota (Bostrom et al., 2001; Haffajee and Socransky, 2001), the differences between the subgingival microbiota in patients from different geographic locations (Haffajee et al., 2004), the relationship of ethnic/racial group, occupational and periodontal disease status (Craig et al., 2001), and effects of different periodontal therapies (Feres et al., 2001; Sakellari et al., 2001). While the technique is rapid, sensitive, and relatively inexpensive, its major shortcoming was cross-hybridization (Socransky and Haffajee, 2005). The implementation of new technologies such as DNA microarrays and NGS improved upon our understanding of the human oral microbiome (Pozhitkov et al., 2011) — but these technologies are not really quantitative.

Gene Meter methodology allows researchers to precisely determine microbial abundances in biological samples. In this study, abundances were used to compare different clinical conditions in order to find abundance signatures for dysbiosis, periodontitis and edentulism. The signatures could be used in future studies, individually or in combination, to assess the clinical status of a patient (e.g., evaluating treatments, such as antibiotic therapies, and oral microbial transplantations, e.g., Pozhitkov et al., 2015). It should be noted that Gene Meter methodology has also been used for the analysis of copy number variation in mice (Pozhitkov et al., 2014a) and postmortem gene expression in zebrafish and mice (Pozhitkov et al., 2016a; Hunter et al., 2016a).

Contributions

Experimental design: PAN and AP.
Laboratory work: MCH, PAN, and AP.
Bioinformatic work: PAN and AP.
Manuscript writing: MCH, PAN, and AP.

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