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ORIGINAL ARTICLE

Predictive modeling of gingivitis severity and susceptibility via oral microbiota

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Predictive modeling of human disease based on the microbiota holds great potential yet remains challenging. Here, 50 adults underwent controlled transitions from naturally occurring gingivitis, to healthy gingivae (baseline), and to experimental gingivitis (EG). In diseased plaque microbiota, 27 bacterial genera changed in relative abundance and functional genes including 33 flagellar biosynthesis-related groups were enriched. Plaque microbiota structure exhibited a continuous gradient along the first principal component, reflecting transition from healthy to diseased states, which correlated with Mazza Gingival Index. We identified two host types with distinct gingivitis sensitivity. Our proposed microbial indices of gingivitis classified host types with 74% reliability, and, when tested on another 41-member cohort, distinguished healthy from diseased individuals with 95% accuracy. Furthermore, the state of the microbiota in naturally occurring gingivitis predicted the microbiota state and severity of subsequent EG (but not the state of the microbiota during the healthy baseline period). Because the effect of disease is greater than interpersonal variation in plaque, in contrast to the gut, plaque microbiota may provide advantages in predictive modeling of oral diseases.

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Subject Category: Microbial population and community ecology

Keywords: gingivitis; plaque; metagenomics; microbial indices of gingivitis

Introduction

With the importance of human microbiota in health and disease being discovered at an unprecedented rate, an ultimate goal has become to classify host states based on the microbiota, and, ultimately, to predict future states based on the current state of the microbiota (Knights *et al.*, 2011; Lozupone *et al.*, 2012). However, thus far, few reports of successful predictive modeling or classification of human disease based on the human microbiota have been published (Faith *et al.*, 2011; Qin *et al.*, 2012). Gingivitis, inflammation of the soft tissues surrounding the teeth,

is one of the most prevalent infections and the most common oral disease in humans. As a worldwide health concern, it affects most children and adolescents (Petersen *et al.*, 2005; Kornman, 2008; Filoche *et al.*, 2010; Jin *et al.*, 2011). The disease is believed to result from buildup of plaque (Moore *et al.*, 1987) and ensuing interactions between the plaque microbiota and host tissues (Handfield *et al.*, 2008; Offenbacher *et al.*, 2009). Although no apical migration of the junctional epithelium occurs, these tissues become erythematous and bleed upon probing. Moreover, chronic gingivitis can progress to periodontitis, an irreversible periodontal infection characterized by alveolar bone loss, attachment loss, formation of periodontal pockets and eventually tooth loss (Sheiham, 1997; Loesche, 2007; Ramseier *et al.*, 2009). Therefore, preventive measures against gingivitis, and improved tools for prognosis and early diagnosis, are of particular clinical significance.

Several factors have hindered investigating the etiology of gingivitis (Tatakis and Trombelli, 2004).

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In natural human populations, gingivitis symptoms can be reversible and volatile, because numerous internally or externally imposed disturbances including oral hygiene practices (personal or professional), impairment of immune system, injury, diet and oral state may all potentially affect disease development and confound disease monitoring (van der Weijden *et al.*, 2002; Sharma *et al.*, 2004). Moreover, clinical diagnoses of gingivitis at present are typically based on individual observations and judgment by human examiners, where the results can be difficult to compare between patients and examiners. Furthermore, despite the complexity of oral microbial communities and the suspected polymicrobial nature of chronic oral infections, most population-wide surveys of gingivitis-associated microbiota have been limited to only a few culturable bacteria (for example, the 'red complex' including *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola*) (Loe *et al.*, 1965; Savitt and Socransky, 1984; Socransky *et al.*, 1998; Haffajee *et al.*, 2008; Igic *et al.*, 2012; Eick *et al.*, 2013) or have employed a small sampling size (Kistler *et al.*, 2013).

To address these challenges, we designed a retrogression–progression model to simulate the development of gingivitis in human population. Fifty human adults underwent a controlled temporal transition from naturally occurring gingivitis at day –21 to healthy gingivae at day 0 ('baseline'), then back to a state of experimental gingivitis (EG) at day 21. For each host, the structure and function of the plaque microbiota was measured at the three time points along the retrogression–progression model: natural gingivitis (NG), baseline and EG. We could thus gain some understanding of the dynamics of the microbiota within each subject, albeit with limited temporal resolution. The taxonomic structures of the plaque microbiota were determined by pyrosequencing of 16S rRNA genes, and functional profiles of the corresponding microbiomes determined by shotgun metagenomic sequencing. Our results suggested that the plaque microbial structure is able to classify gingivitis susceptibility and severity in natural human populations, and that the plaque microbial population during NG can predict the population structure during a later episode of experimentally induced gingivitis in the same subject.

Materials and methods

Study design and sample collection

The experimental model of gingivitis was established as a non-invasive model in humans for understanding pathogenesis of gingivitis (Loe *et al.*, 1965; Offenbacher *et al.*, 2009; Grant *et al.*, 2010; Lee *et al.*, 2012). Experiments were conducted at Procter & Gamble (Beijing) Technology Co., Ltd., Oral Care Department, with approval from the P&G Beijing Technical Center (China) Institutional

Review Board and in accordance with the World Medical Association Declaration of Helsinki (1996 amendment). The International Conference on Harmonisation guidelines for good clinical practice were followed. Fifty volunteers recruited in this study completed both the oral hygiene phase (days –21–0) and the EG phase (days 0–21). For each subject, supragingival plaque along the gumline within 2 mm depth was collected with Gracey curette by qualified dentists at day –21 (NG), day 0 (baseline) and day 21 (EG). To validate the predictive model of Microbial Indices of Gingivitis, an additional cohort of 41 subjects were recruited and analyzed, who also completed the oral hygiene phase and were sampled at day –21 (NG) and day 0 (baseline).

DNA extraction and sequencing

Genomic DNA was extracted from plaque. Barcoded 16S rRNA amplicons (V1–V3 hypervariable region) of all samples were sequenced on 454 Titanium (Branford, CT, USA). For the 18 microbiota of both baseline and EG from 9 subjects that were selected from the 50-subject cohort, total plaque metagenomic DNA was sequenced on Illumina HiSeq 2000 (San Diego, CA, USA).

Full methods and related references are available in Supplementary Information.

Results

An experimentally tractable model of gingivitis retrogression and progression

On day –21 (the NG state), all 50 subjects (17 males and 33 females) exhibited gingival inflammation. In the 50-host cohort, the gingival bleeding (also described as 'Bleeding on Probing') values ranged from 5 to 27, and the Mazza Gingival Index (Materials and methods) ranged from 1.18 to 2.24 (Figure 1). These subjects then underwent a rigorous oral hygiene regimen for 3 weeks, which resulted in greatly decreased gingival bleeding and Mazza Gingival Index values (median gingival bleeding and Mazza Gingival Index were 1.00 and 1.02, respectively) on day 0 (the baseline state), which represented a healthy gingival state. Next, these subjects underwent a 3-week program inducing EG, which resulted in significantly increased gingival bleeding (median 23) and Mazza Gingival Index (median 2.11) at day 21 (the EG state) ($P < 0.01$ for gingival bleeding and Mazza Gingival Index).

At the population level, Mazza Gingival Index ($P < 0.001$) and gingival bleeding ($P = 0.026$) were significantly higher during EG (mean gingival bleeding 26.00 ± 9.59 and Mazza Gingival Index 2.12 ± 0.48) than during NG (mean gingival bleeding 13.5 ± 5.12 and Mazza Gingival Index 1.61 ± 0.24) based on paired *t*-tests. Furthermore, for individual subjects, clinical parameters between NG and EG were significantly correlated. Parameters that were significantly correlated

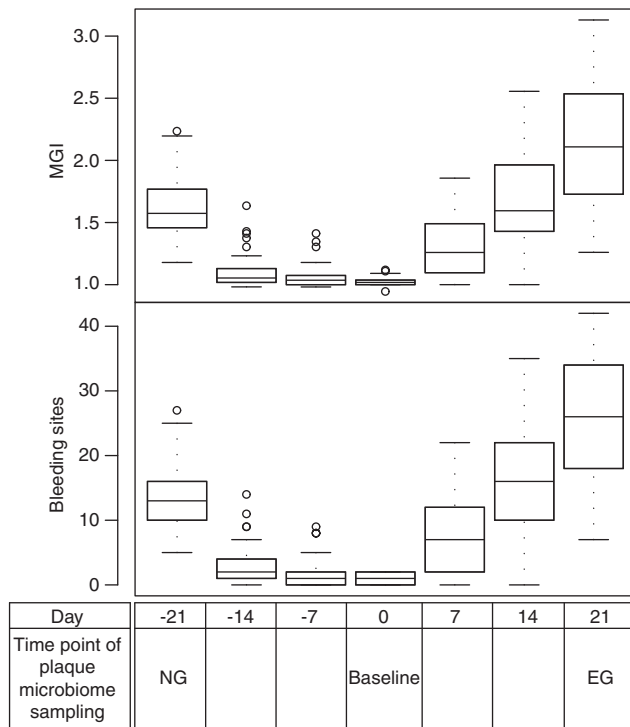


Figure 1 Design of longitudinal study simulating gingivitis development in human population. Boxes represent the inter-quartile range (IQR) and the lines inside represent the median. Whiskers denote the lowest and highest values within $1.5 \times$ IQR. Fifty subjects were recruited for this study over 42 days. At day -21 , all subjects exhibited a certain level of gingival inflammation that represented the state of naturally occurring gingivitis ('NG') with gingival bleeding ranging from 5 to 27 and average Mazza Gingival Index from 1.18 to 2.24. These subjects then underwent rigorous oral hygiene practice for 3 weeks, which resulted in a greatly reduced gingival bleeding and Mazza Gingival Index (median gingival bleeding and Mazza Gingival Index were 1.00 and 1.02, respectively) at 0 day ('baseline') that represented a healthy gum state. Then, the hosts further underwent an oral hygiene program for gingivitis induction for 3 weeks that resulted in significantly increased gingival bleeding (median 23) and Mazza Gingival Index (median 2.11) representing the state of EG.

between the two time points in the same subjects included gingival bleeding (Pearson's correlation: $r = 0.31$, $P = 0.03$) and Mazza Gingival Index (Pearson's correlation: $r = 0.35$, $P = 0.01$).

We have previously shown that plaque microbiota changed more during gingivitis than did the salivary microbiota (Huang *et al.*, 2011). Therefore, to trace the structural and functional dynamics of the bacterial community during gingivitis retrogression and progression, we started by sequencing 16S rRNA gene amplicons from 150 plaque samples (one sample each during NG, baseline and EG for each of the 50 subjects), averaging 7293 sequences per sample (Supplementary Table S1).

The oral microbiota is profoundly altered during the retrogression–progression model

For each of the 150 plaque microbiota, bacterial phyla, genera and species were identified and their

relative abundance quantified via taxonomic assignment against reference databases. At the phylum level, nearly all sequences were from 13 bacterial phyla, including 6 predominant bacterial phyla commonly encountered in the oral cavity: *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Fusobacteria* and TM7 (each with average relative abundance $>1\%$ at least one time point) (Supplementary Figure S1A). Between the gingivitis states (NG and EG) and the healthy gingival state (baseline), significant difference ($P < 0.05$; paired *t*-test) was found in five predominant phyla: *Actinobacteria*, *Firmicutes*, TM7, *Bacteroidetes* and *Fusobacteria*. A temporal shift of community structure along the progression from NG to baseline to EG was apparent, characterized by the elevated relative abundance of *Actinobacteria* and *Firmicutes* at baseline, and that of TM7, *Bacteroidetes* and *Fusobacteria* during NG and EG (Supplementary Figure S1A).

At the genus level, 27 bacterial genera (each with average relative abundance $>0.1\%$ at least one time point) were differentially distributed ($P < 0.05$, paired *t*-test; false discovery rate $q < 0.2$) between baseline and gingivitis (both NG and EG). Among them, 5 (*Streptococcus*, *Rothia*, *Actinomyces*, *Haemophilus* and *Lautropia*) showed elevated abundance at baseline, while 22 (*Leptotrichia*, *Prevotella*, *Fusobacterium*, TM7 genus, *Porphyromonas*, *Tannerella*, *Selenomonas*, uncultured *Lachnospiraceae*, unclassified *Comamonadaceae*, *Peptococcus*, *Aggregatibacter*, *Catonella*, *Treponema*, SR1 genus, *Campylobacter*, *Eubacterium*, *Peptostreptococcus*, unclassified *Bacteroidaceae*, *Solobacterium*, *Johnsonella*, *Oribacterium*, and unclassified *Veillonellaceae*) were enriched in both NG and EG (Supplementary Figure S1B). During the retrogression–progression model, different bacterial species within the same genus usually exhibited identical patterns of relative-abundance change, except for several species of *Capnocytophaga*, *Actinomyces* and *Streptococcus* (Supplementary Figure S1C).

Structural and functional features of gingivitis-associated microbiota

To identify features of the microbiota associated with gingivitis, all 150 healthy and diseased microbiota were clustered via principal component analysis (PCA) based on the relative abundance of genus-level taxa (Figure 2a). We tested whether procedures previously used to attempt to describe enterotype clustering in the human gut would identify natural clusters in our oral data. Partitioning Around Medoids clustering analysis (natural clustering of the 150 microbiota based on their structure; Materials and methods) did not support the presence of 2 clusters in the 150 microbiota (Silhouette value = 0.31; no cluster formation was observed within the 100 diseased microbiota either;

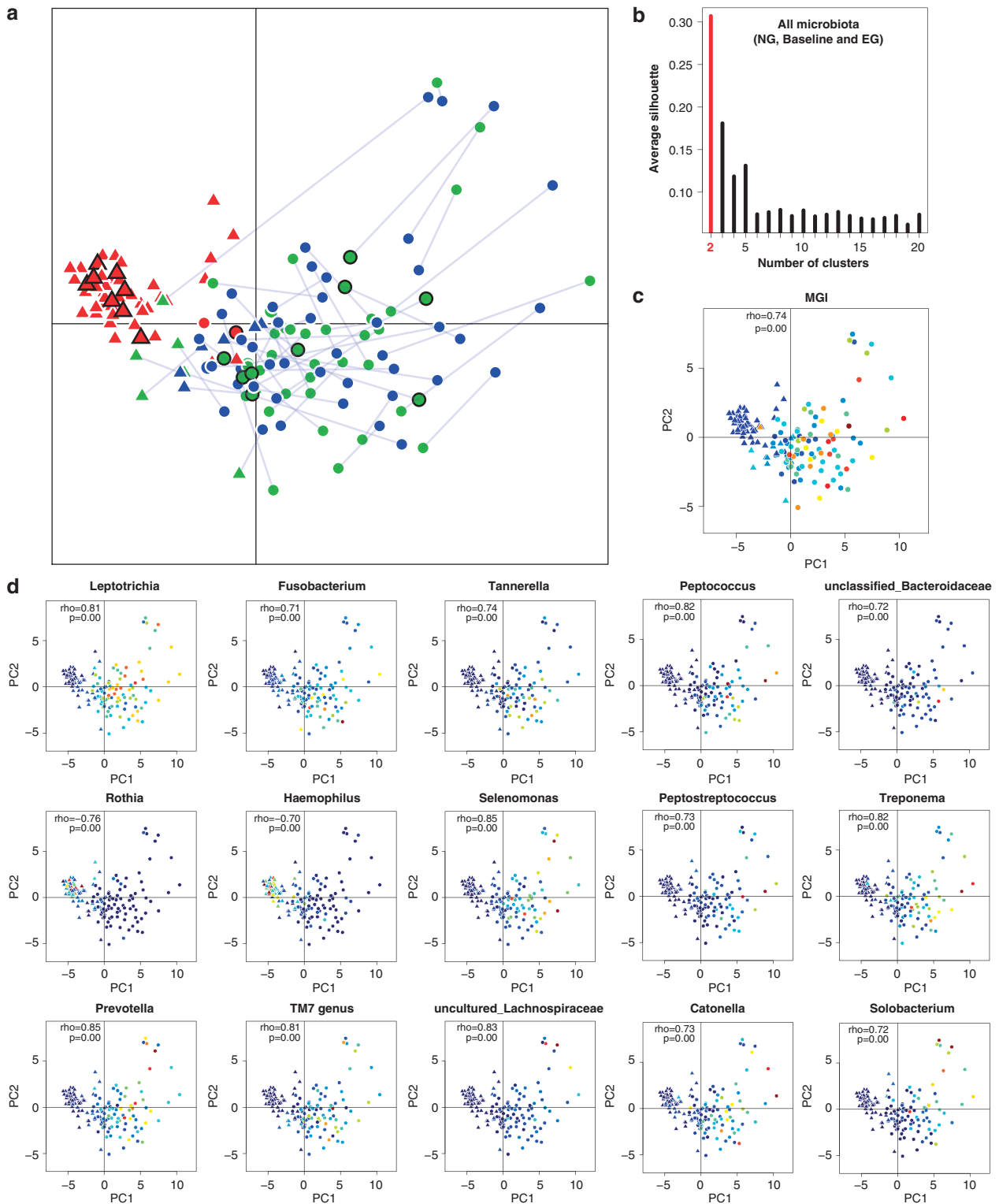


Figure 2 Structural features of healthy and gingivitis microbiota. **(a)** Distinction in organismal structure between healthy (red) and gingivitis-associated plaque microbiota (blue and green). Microbiota from the same host were connected by solid lines between NG (blue) and EG (green). Those selected for functional analysis using whole-metagenome sequencing were labeled (black circled points). Triangle and dots indicated samples from the two putative clusters based on the Partitioning Around Medoids (PAM) clustering. **(b)** PAM clustering method using Jensen-Shannon distance argued against the presence of two or more clusters in all microbiota or in the diseased microbiota. (The x axis shows cluster number; the y axis shows CH index, a measure of cluster separation.) All samples were plotted on the first two principal components of the genus profile. Host and microbial features of each of the 50 human individuals were shown in the same plot as **(a)**: gradients of Mazza Gingival Index **(c)** and abundance of all 15 driver genera identified **(d)** are colored from low value/abundance (blue) to high value/abundance (red).

Figure 2b; Koren *et al.*, 2013). However, the healthy and diseased microbiota were largely concentrated along a boundary separating baseline samples from both NG and EG (Figure 2a), suggesting that microbiota structure is connected to disease state and can classify individuals according to disease state. Procrustes analysis revealed that, between healthy gums (baseline; Mazza Gingival Index <1.10) and gingivitis-active gums (NG and EG; Mazza Gingival Index >1.15), microbiota structures were not statistically significantly associated by subject, that is, the relative orientations of samples from the same subject in the two groups of points (NG to baseline and baseline to EG) were not associated (Figure 2a; $P=0.07$ for NG versus baseline; $P=0.21$ for baseline versus EG; 10 000 Monte Carlo label permutations per experiment). However, despite the higher Mazza Gingival Index and gingival bleeding during EG, within-subject structures between NG and EG were largely consistent ($P<0.001$ for NG versus EG by 10 000 Monte Carlo label permutations; Figure 2a; Materials and methods), suggesting that microbial community perturbations associated with gingivitis recur the same way in the same subjects and therefore that the community configuration during the earlier episode of gingivitis can predict relapse to broadly the same community during the later episode. These results were also supported by principal coordinates analysis based on UniFrac (Hamady *et al.*, 2010) and ThetaYC distances (Supplementary Figure S2). Consequently, each subject may have a personalized disease-associated configuration of the microbiota that recurs over time.

The current clinical practice of separating hosts into diseased and healthy groups was based on the arbitrary Mazza Gingival Index threshold of 1.10–1.12. However, such a bimodal definition of health and disease does not match the observed characteristics of hosts and microbiota. In fact, the distribution of clinical parameters (for example, Mazza Gingival Index, Materials and methods) both within individual hosts and in human populations was continuous (Figure 1). PCA suggested that the transition of the microbiota between NG, baseline and EG was not a discrete process, but rather gradient-like (Figures 2a and c). Therefore, a new clinical model is required that considers the distribution of both disease phenotype and microbiota structure along a gradient, which should also be useful for providing a more objective measure of disease states and allowing more appropriate statistical tests of links between the microbiota and the disease.

The projected coordinate of a given microbiota on the first principal component (PC1) appeared to capture the gradient-like heterogeneity and development of microbiota structure along disease retrogression and progression, because changes in PC1 within subjects and across cohorts were largely consistent with the structural segregation between healthy and diseased microbiota (Figure 2a).

Moreover, the relative order of microbiota along PC1 defined using all 150 samples (Figure 2a) was similar to those defined using only healthy, only NG or only EG microbiota alone (Spearman correlation; all versus healthy-only: $\rho=0.95$, $P<0.001$; all versus NG: $\rho=0.97$, $P<0.001$; all versus EG: $\rho=0.97$, $P<0.001$, Materials and methods). Therefore, PC1 appeared to be the primary descriptor and a good proxy for quantitatively measuring the development of the microbiota during both transitions (NG to baseline and baseline to EG).

For the 50 hosts considered at all three time points, 15 bacterial genera were found to be the drivers of microbiota heterogeneity along PC1, as their gradients in abundance were significantly correlated with the coordinates of their corresponding samples on PC1 (Figure 2d; Spearman $\rho>0.7$, false discovery rate $q<0.2$). These drivers included *Rothia*, *Haemophilus*, *Prevotella*, *Leptotrichia*, *Fusobacterium*, *Selenomonas*, uncultured *Lachnospiraceae*, TM7 genus, *Tannerella*, *Peptococcus*, *Peptostreptococcus*, *Catonella*, *Treponema*, *Solobacterium* and unclassified *Bacteroidaceae*. Two of the fifteen genera, *Rothia* and *Haemophilus*, decreased in relative abundance along PC1 ('negative drivers'), while the other thirteen increased along PC1 ('positive drivers'; Figure 2d).

To test the functional features of gingivitis microbiota, the genomic DNA from 18 of the plaques (from 9 of the subjects, each of whom was sampled during both baseline and EG) was extracted and shotgun sequenced respectively at a depth of averagely 3.94 Gb per sample (Table 1; Materials and methods). These nine subjects were picked to maximize the phylogenetic diversity of microbiota sampled (their relative coordinates shown on the PCA plot of all 150 samples in Figure 2a). Functional genes encoded in the microbiota were analyzed based on Clusters of Orthologous Groups (COGs) database (Tatusov *et al.*, 2001) and compared based on the relative frequencies of the assigned COGs (Materials and methods). Interestingly, Procrustes analysis (Muegge *et al.*, 2011) indicated that, among the 18 samples, the agreement between phylogenetic and functional measurements based on COGs was excellent ($P<0.001$ using 10 000 Monte Carlo label permutations; Figure 3a). Furthermore, clustering of the 18 microbiota based on encoded functional genes, nearly identical to that based on the organismal structure, suggested that microbiota differed in functional gene structure between healthy subjects and those with gingivitis (Figure 3b). In total, 1205 COGs involving 24 functional categories (out of 4873 COGs in 25 categories) were either positively or negatively gingivitis associated ($P<0.01$) (Supplementary Table S2). For example, in Functional Category N (cell motility), 33 COGs mostly related to flagellar biosynthesis pathways were enriched in gingivitis, while merely 3 COGs (all related to pilus assembly protein) were enriched in healthy hosts (Figure 3c). On the other hand, in

Table 1 Features of metagenome shotgun sequences produced for the 18 plaque microbiota

Host ID	Age	Sex	Baseline				EG (experimentally induced gingivitis)					
			Sample ID	Mazza GI	Bleeding sites number	Metagenome sample size (Gb)	Metagenome reads	Sample ID	Mazza GI	Bleeding sites number	Metagenome sample size (Gb)	Metagenome reads
9066	32	F	9066B	1.00	0	3.92	26 110 020	9066E	1.93	26	4.05	26 999 772
9174	36	F	9174B	1.05	2	3.75	25 005 760	9174E	2.63	39	4.14	27 567 214
9183	26	M	9183B	1.02	1	4.15	27 644 386	9183E	1.77	19	3.91	26 036 894
9439	27	F	9439B	1.00	0	3.73	24 860 734	9439E	1.68	19	4.08	27 193 368
9445	27	M	9445B	1.05	2	3.25	21 661 610	9445E	1.98	26	4.08	27 186 152
9147	28	M	9147B	1.04	2	3.99	26 585 026	9147E	2.34	29	3.96	26 410 660
9148	41	F	9148B	1.07	2	4.02	26 815 920	9148E	3.13	41	3.27	21 798 906
9307	34	F	9307B	1.04	2	4.14	27 567 214	9307E	2.33	32	4.21	28 041 294
9325	32	F	9325B	1.00	0	4.14	27 590 228	9325E	2.52	36	4.09	27 281 026

Abbreviation: Mazza GI: Mazza Gingival Index.

Functional Category P (inorganic ion transport and metabolism), 32 COGs were enriched in healthy samples while only 19 were depleted. Thus, gingivitis microbiomes were distinct from healthy ones in both structure and function.

Link between PC1 and disease phenotype

The classification of healthy and diseased microbiota using the PCA based on the 16S rRNA gene taxonomy was identical to that using the PCA based on functional genes, suggesting that the value of each sample along the PC1 axis is a useful descriptor for both structural and functional features of gingivitis microbiota.

The value of PC1 appeared to harbor clinically useful information. During NG (and also during EG), there was a significant correlation between Mazza Gingival Index and PC1 values among the 50 subjects (Table 2; Spearman correlation NG: $\rho=0.37$, $P<0.01$; EG: $\rho=0.48$, $P<0.001$). Moreover, between NG and baseline (and also between baseline and EG), the PC1 values of the 100 microbiota were positively correlated with Mazza Gingival Index (Spearman correlation; all: $\rho=0.74$, $P<0.001$; NG to baseline: $\rho=0.77$, $P<0.001$; baseline to EG: $\rho=0.79$, $P<0.001$).

Change in PC1 was also clinically relevant. Among the 50 hosts, in each of the 2 segments within the retrogression–progression model (NG to baseline and baseline to EG), the within-subject changes in PC1 and Mazza Gingival Index were significantly correlated (Table 2; labeled in *italic*), as were the correlations between the changes in PC1 between the two segments, the changes in Mazza Gingival Index between the two segments (Table 2; labeled in **bold**). Moreover, the within-subject change in PC1 was significantly correlated with the within-subject change in Mazza Gingival Index between NG and EG (Table 2; Spearman correlation $\rho=0.56$, $P=0$). Interestingly, for the 10 bottom-quintile subjects with little change in Mazza Gingival Index between NG and EG, the

change in PC1 was not significantly correlated with the change in Mazza Gingival Index (Spearman correlation $\rho=0.25$, $P=0.48$). However, for the 10 top-quintile subjects where the Mazza Gingival Index changed the most between NG and EG, the change in Mazza Gingival Index was significantly correlated with the change in PC1 (Spearman correlation $\rho=0.64$, $P=0.05$), suggesting that the change in PC1 quantitatively reflects the degree of change in gingivitis symptoms.

Two types of hosts with distinct sensitivity to gingivitis

Among the 50 subjects, most hosts exhibited a largely consistent microbiota structure during the disease progression from NG to EG (Figure 2a). Although changes in PC1 associated with the transition from NG to baseline (or baseline to EG) varied considerably among the 50-host cohort, the rate of microbiota change from NG to baseline and that from baseline to EG was largely similar within each subject (Table 2; Figure 4a). The rate of Mazza Gingival Index change followed a similar pattern (Table 2; Figure 4a). Furthermore, the gingivitis severity (that is, Mazza Gingival Index) during EG was highly correlated with that during NG, as was microbiota structure (that is, PC1) (Table 2). The persistence of disease outcome as well as microbiota structure for majority of the hosts during EG (as compared with NG) suggested the presence of host-dependent (and likely personal) factors in determining the susceptibility to gingivitis recurrence in natural human populations.

To test whether disease susceptibility differed among the 50 subjects, we performed a PCA using as input variables the change along PC1 from NG to baseline and from baseline to EG, and the change in Mazza Gingival Index in each of these two segments, for each subject (Figure 4b). The distribution pattern of the 50 hosts suggested a bimodal distribution ($P=0.74$ for the hypothesis of non-bimodal distribution based on Hartigan's dip test for unimodality),

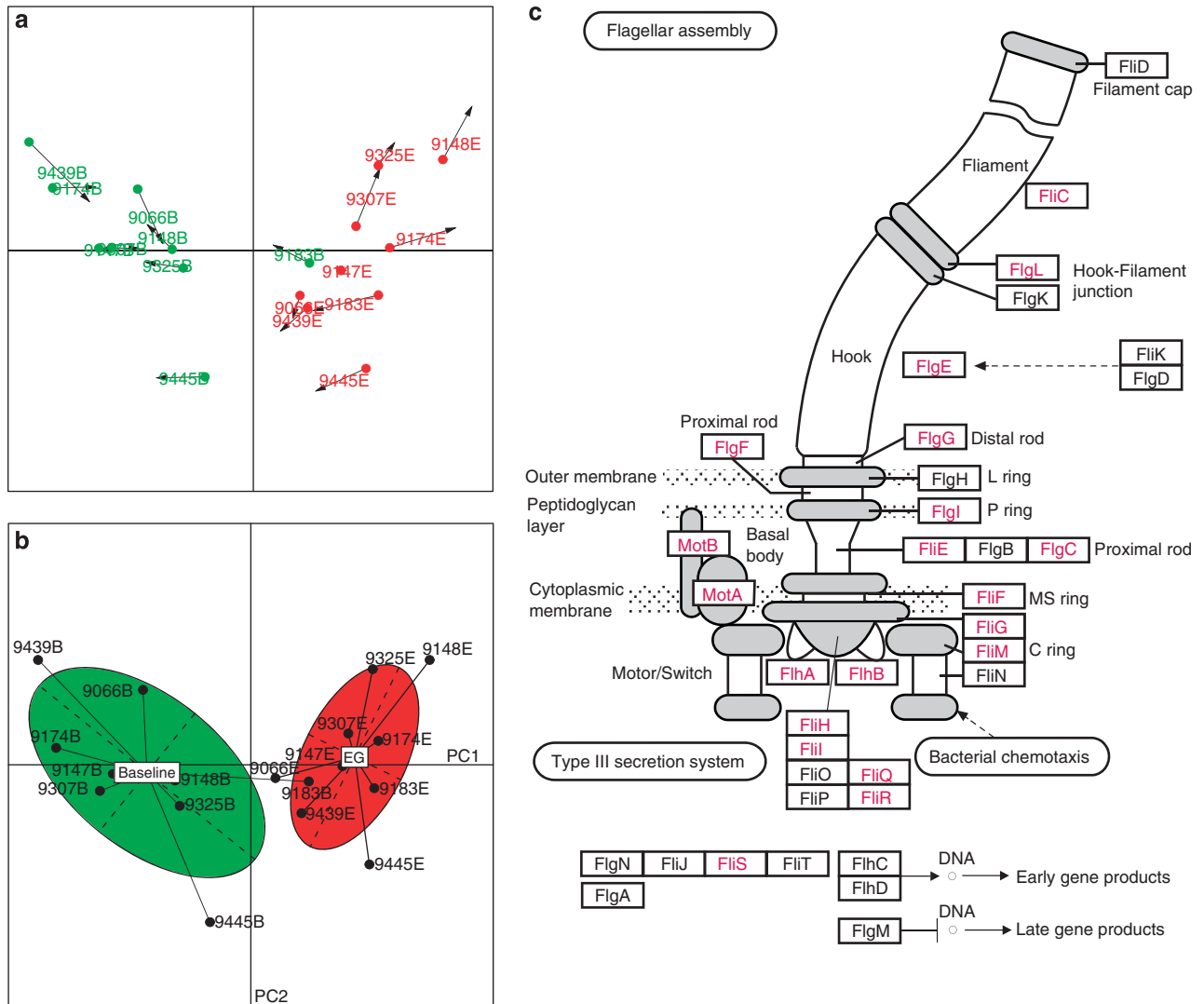


Figure 3 Functional features of gingivitis-associated microbiota. **(a)** Procrustes analysis of 16S rRNA gene sequences (PCA) against COGs. Each point represented a plaque microbiota and was colored according to the clinical status. The arrow end of each line connected to the 16S rRNA data for the sample, whereas the other end connected to the functional annotation. The fit of each Procrustes transformation over the first four dimensions was reported as the P -value by 10 000 Monte Carlo label permutations. **(b)** Functional distinctions between healthy and gingivitis microbiota. PCA showed that disease state significantly affected the microbial functional structure. The effects of disease state on the microbial functions appeared to be well separated by the first axis. **(c)** The 33 gingivitis-enriched orthologous groups that encode components of the flagellar biosynthesis pathway. The schematic was adapted from KEGG, with gene names of the corresponding KO (KEGG Ortholog) highlighted in red.

where a discriminating line can be drawn to divide the hosts into two types (Figure 4b), which we designated as type I (17 individuals) and type II (33 individuals). Type-II hosts were characterized by more acute changes in both microbiota structure and Mazza Gingival Index than type-I hosts (Figure 4c). For an average type-II host, the rate of change in PC1 was 0.33 per day, and the rate of change in the Mazza Gingival Index 0.05 per day, which were respectively 2.21-fold and 1.89-fold of an average type-I host (Figure 4c).

During both NG and EG, there were significant relationships between these types of host sensitivity to gingivitis and the relative abundance of certain taxa ($P < 0.05$, Wilcoxon rank-sum test). These taxa

included *Abiotrophia*, *Selenomonas*, *uncultured Lachnospiraceae*, *Peptococcus*, *uncultured Bacteroidaceae*, *Peptostreptococcus*, *Oribacterium* and *uncultured Veillonellaceae*; all were enriched in type-II hosts as compared with type-I hosts, except *Abiotrophia* which was enriched in type I (Figure 4d). Most (five) of these type-II hosts associated genera were among the fifteen drivers of PC1.

Interestingly, compared with type-I hosts, those genera enriched in type-II hosts at NG and EG were also higher in relative abundance in type-II hosts at baseline. Thus, the heterogeneity of plaque microbiota among hosts may explain at least partially, either as a cause or as a consequence, the interhost phenotypic variations of gingivitis sensitivity and

Table 2 Correlation between alteration in microbiota structure (Δ PC1) and change in MGI (Δ MGI) for the 50-host cohort at NG-baseline, baseline-EG and NG-EG

Spearman correlation (ρ)	NG to baseline				Baseline to EG				NG to EG			
	NG.PC1	Δ PC1	NG.MGI	Δ MGI	B.PC1	Δ PC1	B.MGI	Δ MGI	EG.PC1	Δ PC1	EG.MGI	Δ MGI
<i>NG to baseline</i>												
NG.PC1	NA	—	—	—	—	—	—	—	—	—	—	—
Δ PC1	-0.86	NA	—	—	—	—	—	—	—	—	—	—
NG.MGI	0.37	-0.42	NA	—	—	—	—	—	—	—	—	—
Δ MGI	-0.36	0.4	-0.98	NA	—	—	—	—	—	—	—	—
<i>Baseline to EG</i>												
B.PC1	—	—	—	—	NA	—	—	—	—	—	—	—
Δ PC1	0.28	-0.51	—	—	—	NA	—	—	—	—	—	—
B.MGI	—	—	—	—	—	—	NA	—	—	—	—	—
Δ MGI	—	—	0.39	-0.38	—	0.53	—	NA	—	—	—	—
<i>NG to EG</i>												
EG.PC1	0.4	-0.44	—	—	—	0.89	—	0.48	NA	—	—	—
Δ PC1	-0.56	0.43	—	—	—	0.49	—	0.43	0.43	NA	—	—
EG.MGI	—	—	0.39	-0.37	—	0.53	—	1	0.48	0.43	NA	—
Δ MGI	—	—	—	—	—	0.44	—	0.86	0.41	0.56	0.86	NA

Abbreviations: EG, experimental gingivitis; MGI, Mazza Gingival Index; NG, natural gingivitis; PC1, first principal component. Only the ρ values with its corresponding $P < 0.05$ (that is, significant correlation) were shown. Significant within-phase Δ PC1- Δ MGI correlations were labeled in italic. Significant inter-phase Δ PC1- Δ PC1 or Δ MGI- Δ MGI correlations were labeled in bold.

possibly susceptibility to disease reoccurrence in human populations.

Microbial Indices of Gingivitis

The strong correlation between PC1 and disease symptom (Mazza Gingival Index) both between subjects and within subjects thus suggested that PC1 could potentially model disease progression, and classify subjects according to their disease state. To test this hypothesis, the 50-host cohort was used as a training set for model construction, and an additional 41 human subjects with naturally occurring gingivitis were recruited and then each sampled during both NG and baseline (thus 82 additional microbiota samples were sequenced) for model validation.

(1) *MiG27*: We derived a ‘microbial index of gingivitis’ (MiG) based on the relative abundance of the 27 bacterial markers that distinguish between the baseline stage and the gingivitis stages (NG and EG) in the 50-host cohort (*MiG27*; Supplementary Figure S1B; Table 3):

$$\text{MiG27} = \left(\frac{\sum_{i=22} \text{adundance}(g_{\text{gingivitis enriched}})_i}{22} - \frac{\sum_{j=5} \text{adundance}(g_{\text{health enriched}})_j}{5} \right) \times 10$$

In the 50-host cohort, *MiG27* was highly correlated with Mazza Gingival Index during the transition both from NG to baseline ($P < 0.001$, Student’s *t*-test) and from baseline to EG ($P < 0.001$, Student’s *t*-test): the area under the receiver

operating characteristic curve was 99.52% (95% confidence interval (CI): 98.77 – 100%) for the NG to baseline transition, and 99.84% (95% CI: 99.53 – 100%) for the baseline to EG transition (Supplementary Figure S3A). The predictive power of *MiG27* was tested by predicting the gingivitis status of the 41 hosts in the 41-host cohort using their NG microbiota. The *MiG27* between NG (Mazza Gingival Index > 1.18) and baseline (Mazza Gingival Index < 1.12) was significantly different ($P < 0.001$, paired *t*-test), for example, the top 27 samples with the highest *MiG27* were all correctly classified as gingivitis (Supplementary Figure S3B). The overall accuracy of classification (based on Linear Discriminant Analysis) for diseased state versus healthy state is 94% (that is, an error rate of 6.1%) (Table 3). Thus, *MiG27* might be valuable for screening for gingivitis in clinical settings.

(2) *MiG15*: Although *MiG27* distinguishes between health and gingivitis with high accuracy, a classifier system for disease severity in gingivitis population would be useful. Thus, we derived *MiG15*, which was based on the relative abundance of 15 bacterial genera that drive the structural heterogeneity of microbiota along PC1 (Table 2):

$$\text{MiG15} = \left(\frac{\sum_{i=13} \text{adundance}(g_{\text{High_PC1 enriched}})_i}{13} - \frac{\sum_{j=2} \text{adundance}(g_{\text{Low_PC1 enriched}})_j}{2} \right) \times 10$$

The *MiG15* could differentiate gingivitis and health for 41 validation subjects with high accuracy

as MiG27 (Figure 5a; Table 3). We then regressed the relative PC1 values (Y : the development of gingivitis) on MiG15 (X) using linear regression. The regression formula is $Y = -0.97 - 4.62X$. This

revised model accounted for 60% of variance in PC1 location in the 50-host cohort. The predictive power of this model on disease severity was tested based on the microbiota during NG in the 41-host cohort.

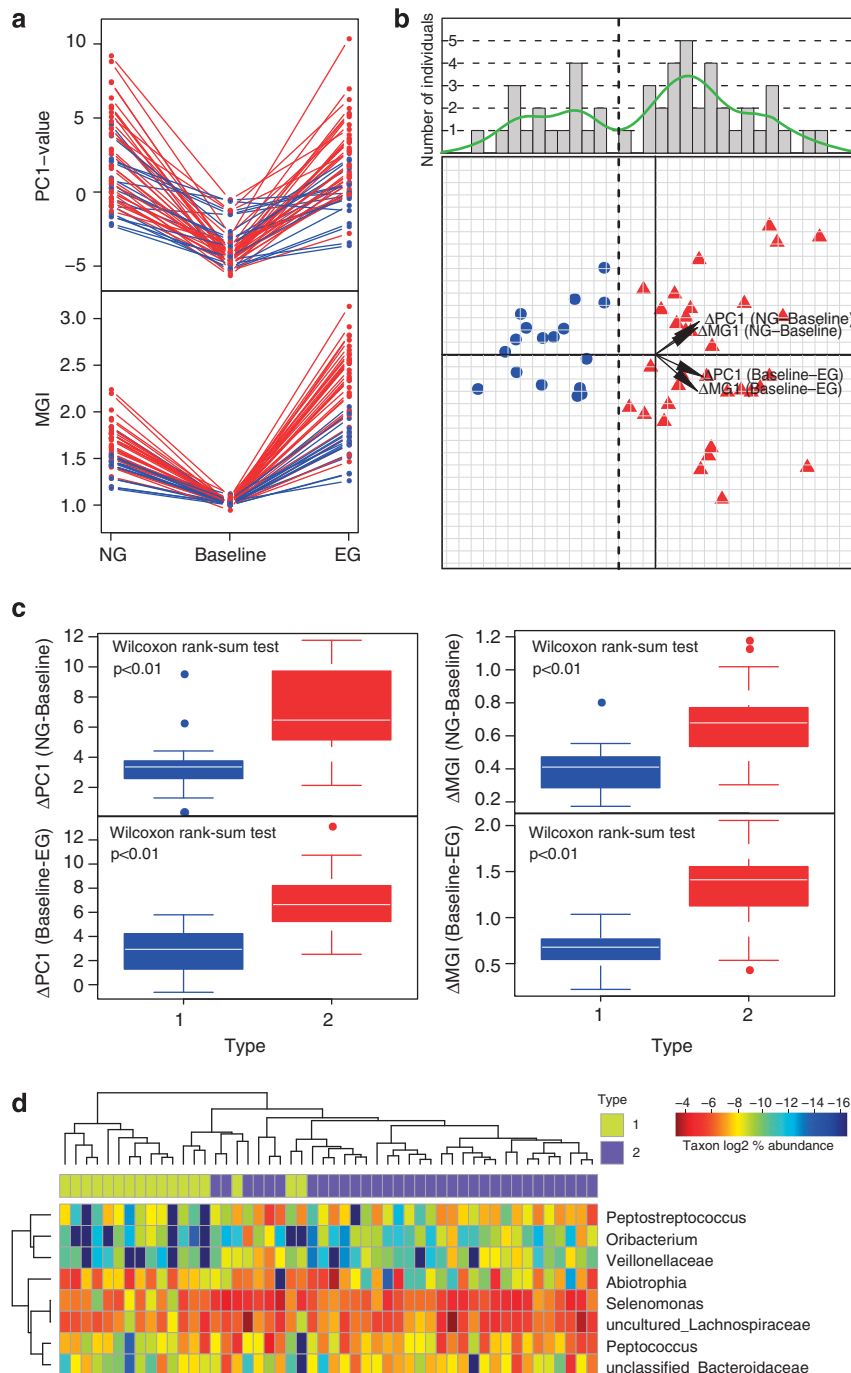


Figure 4 Heterogeneity of gingivitis sensitivity in human hosts. **(a)** The patterns of microbiota structural (that is, PC1 values) change and Mazza Gingival Index change during the retrogression–progression model. The two colors indicated type-I (blue) and type-II (red) hosts. **(b)** All hosts in the 50-member cohort were plotted on the first two principle components of the PCA based on the change profiles of microbiota and Mazza Gingival Index. The histogram and the kernel density plot (green line) describing distribution of the 50 hosts along the principle component of the PCA were shown. The vertical dash line divided the 50 hosts into type I (blue) and type II (red). The four variables as main contributors to these clusters were determined and plotted by their loadings in these two principal components. **(c)** Difference in gingivitis sensitivity between type-I and type-II hosts. The within-subject Δ PC1 and Δ Mazza Gingival Index were compared. Boxes represented the interquartile range (IQR) and the lines inside represent the median. Whiskers denoted the lowest and highest values within $1.5 \times$ IQR. **(d)** Bacterial genera associated with type I and type II of hosts with distinct disease sensitivity. Green: type I enriched; Red: type I depleted.

Categorization of both inferred values and test values (of PC1) into three tertiles revealed an error rate at 24.4% (Figure 5b), suggesting ~75%

Table 3 Predictive models of human gingivitis based on plaque microbiota

Error rate	MiG27 (%)	MiG15 (%)	MiG-S (%)
Clinical status			
Health versus gingivitis	6.10	6.10	—
Categorized status of gingivitis			
Based on MGI	41.50	41.50	—
Based on PC1	24.40	24.40	—
Gingivitis sensitivity of the host			
Based on change pattern of PC1 and MGI	—	—	26.00

Abbreviations: MGI, Mazza Gingival Index; MiG-S, microbial index of gingivitis sensitivity; MiG, microbial index of gingivitis; PC1, first principal component.

classification accuracy of gingivitis severity in natural human hosts (Table 3).

(3) *MiG Sensitivity (MiG-S)*: Furthermore, we derived a ‘microbial index of gingivitis sensitivity’ (MiG-S) based on the relative abundance of the eight bacterial markers that distinguish between the type-I and type-II hosts in the 50-host cohort during NG (MiG-S; Figure 5c; Table 3):

$$\text{MiGS} = \left(\frac{\sum_{i=7} \text{adundance}(g_{\text{TypeII enriched}})_i}{7} - \frac{\sum_{j=1} \text{adundance}(g_{\text{TypeI enriched}})_j}{1} \right) \times 10$$

In the 50-host cohort, MiG-S was highly correlated with types ($P < 0.05$, Wilcoxon rank-sum test): the area under the receiver operating characteristic curve was 74.0% (95% CI: 60.2–87.8%) (Figure 5c), suggesting a 74.0% accuracy for classifying gingivitis-sensitivity host types.

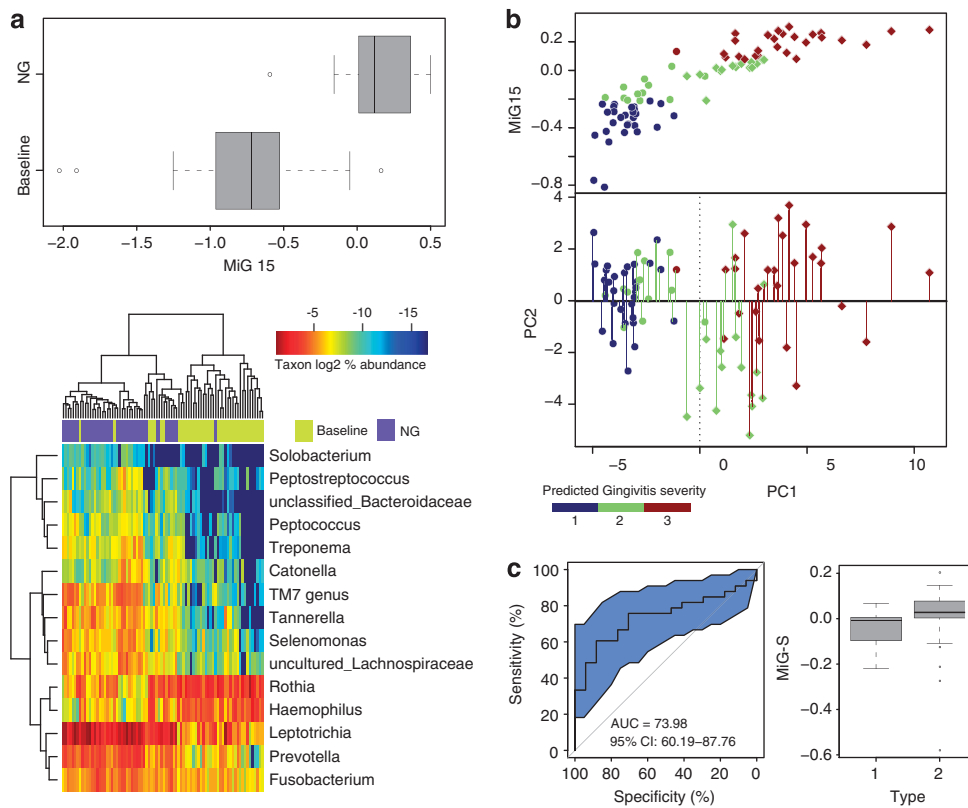


Figure 5 Trial prediction of disease outcome using Microbial Indices of gingivitis (MiGs). (a) The MiG15 indices of an additional cohort of 41 hosts. Boxes represented the interquartile range (IQR) and the lines inside represent the median. Whiskers denoted the lowest and highest values within $1.5 \times \text{IQR}$. The heatmap indicated the ability of MiG15 to discriminate healthy and gingivitis status of hosts. (b) Accuracy of MiG15-based prediction of disease severity in the additional cohort of 41 hosts. All samples in the test cohort were plotted on the first two principal components of the genus profile (point shape of individuals determined to have the same time point: circle: baseline; diamond: NG). Categorization of the actual and predicted PC1 values into three quantiles (blue: 1st quantile; green: 2nd quantile; brown: 3rd quantile) revealed an error rate of prediction at 24.4%. Color of each point showed its predicted PC1 value (that is, n th quantile), while color of the line connected to the point indicates its actual PC1 level (n th quantile). (c) Use of MiG-S to predict the gingivitis-sensitivity type for each host in the 50-member cohort during NG. The accuracy of MiG-S was measured by AUC, which was the area under the receiver operating characteristic (ROC) curve of plaque microbiota-based (that is, MiG-S-based) gingivitis-sensitive host-type classification. The black bars denoted the 95% CI and the blue area between the two outside curves represented the 95% CI shape. The MiG-S index was computed for each host.

Discussion

Our retrogression–progression model of gingivitis revealed source of the heterogeneity of gingival microbiota both within subject and in natural populations. In neither case is there a clear boundary between health and disease in host or microbial attributes: their distribution, as well as retrogressive or progressive succession, was not a discrete but rather a gradient-like process. The developmental program between the healthier and the more diseased states was primarily driven by 15 bacterial genera, most of which increased in relative abundance (except two which decreased) along the development. The taxonomic shift of microbiota was accompanied by a functional shift: the observed gingivitis-enriched functions such as flagellar biosynthesis might be traced to bacterial oral mobility, as the flagellar can assist invading host tissues and escaping phagocytosis (Siqueira and Rocas, 2007). For example, *Treponema*, *Selenomonas* and *Campylobacter*, which were among the gingivitis biomarkers in our MiG model, were among the major donors of flagellar biosynthesis pathways in the plaque microbiota of periodontal disease (Wang *et al.*, 2013); in fact, many species in these genera are equipped with flagella (Ruby *et al.*, 1997; Ihara *et al.*, 2003; Liu *et al.*, 2010; Haya *et al.*, 2011).

Our study also unraveled a microbial basis for the heterogeneity of disease outcome in human population. Two host types (type I and type II) with distinct sensitivity/susceptibility to gingivitis were present, with type-II hosts featuring averagely over two times more acute disease development than type-I hosts. Moreover, gingivitis recurrence appeared personalized, as the gingivitis severity (for example, Mazza Gingival Index) during EG was highly correlated with that during NG, while the disease progression rate (baseline to EG) was highly correlated with the disease retrogression rate (NG to baseline). We have identified a microbial link to the two host types, with eight bacterial taxa specifically associated (seven enriched and one depleted) with type-II hosts during each of NG, baseline and EG. However, because such association between taxa and host types actually persisted even at baseline (that is, 'healthy' state), microbial factors likely have prominent roles in host-type formation, and it is possible that type-II hosts were predisposed to gingivitis reoccurrence due to their residential microbiota during NG. Testing whether bacterial markers during baseline might predict susceptibility to future gingivitis remains an intriguing possibility for future follow-up studies.

Uncovering these major sources of variation in gingival microbiota might have implications for the diagnosis and treatment of periodontal disease. Gingivitis can advance to periodontitis, which is a major cause of tooth loss in adults (Williams, 1990). However, the role of gingivitis in periodontitis pathogenesis remains controversial: an etiological

connection between them has been postulated but not yet proved. One confounding factor has been that not all gingivitis cases proceed into periodontitis: epidemiological studies showed that ~50% of adults have gingivitis around more than six teeth (Oliver *et al.*, 1998), while only 15% of adults suffer from periodontitis (Oliver *et al.*, 1991). In our identified 'gingivitis-driver' genera, several species (for example, *Tannerella forsythia*, *Peptostreptococcus micros* (*Parvimonas micra*), *Fusobacterium nucleatum subsp.*, *Haemophilus paraphrophilus* and *Capnocytophaga* sp. oral clone CZ006 *et al.*) were reportedly associated with periodontitis (Griffen *et al.*, 2012; Tanner *et al.*, 1998; Tanner *et al.*, 2006). In addition, those potential markers of severe gingivitis we identified (for example, *Tannerella*, *Treponema* species and the TM7 phylum) were reportedly enriched in periodontitis (Griffen *et al.*, 2012). Moreover, several potential markers of type-II hosts (for example, *Selenomonas*, *Peptostreptococcus*, unclassified *Lachnospiraceae*, unclassified *Veillonellaceae* and *Oribacterium*), who exhibited higher disease acuteness and susceptibility to recurrence, were also found to be enriched in periodontitis (Griffen *et al.*, 2012). Furthermore, a recent study reported a functional link in oral microbiota between gingivitis and periodontitis patients (Wang *et al.*, 2013). Therefore, the collective evidence supported a link between severe gingivitis and periodontitis, and also provided a possible explanation of the variation of periodontitis susceptibility in human populations.

Finally, the microbial drivers of gingivitis development and susceptibility identified here might provide novel opportunities to improve clinical practice. In gingivitis, the gingival tissue exhibited color change, contour alteration, increased sulcular exudates and bleeding upon provocation (Mariotti, 1999). On the basis of one or more of such host symptoms, current gingival indices proposed or practiced can be subjective and heavily dependent upon the human examiner's visual observation and individual judgment, leading to poor reproducibility among examiners. Moreover, because symptom of gingivitis can vary greatly among different teeth (and even probing points), manually testing two probing sites for each of the 28 teeth for each patient can be time and labor intensive. These drawbacks have collectively confounded cross-examiner and cross-patient analysis of gingivitis. In this study, we have developed and validated an alternative and likely complementary measure for gingivitis that was based on quantitative analysis of plaque microbiota. Our proposed MiG-based predictive models were able to predict diseased microbiota at 95% accuracy, distinguish different disease stages with 75% accuracy, and potentially predict disease sensitivity. With the development of sequencing technology, microbiome analysis could serve as an objective, sensitive and cost-efficient measure of gum health and gingivitis susceptibility and thus

contribute to the diagnosis, prognosis and intervention of gum diseases.

The potential of human microbiota as venues in tracking and diagnosing host conditions (diseases, diets and so on) is dependent on, and limited by, the degree of heterogeneity in microbiota-condition link at the population level. In the gut, differences in the structure of the microbiota structure between hosts appear to be larger than variation associated with clinical conditions (for example, the same subjects during weight loss, or switched between a normal or high-fat diet, tend to resemble themselves over time rather than clustering with others of the same clinical state) (Turnbaugh *et al.*, 2009; Caporaso *et al.*, 2011; Wu *et al.*, 2011). However, our results here revealed that the opposite appears to be true for oral microbiota: differences between healthy and diseased oral microbiota within a subject are larger than interpersonal differences, so that the same person's samples do not resemble themselves over time but instead cluster with samples from other people with the same clinical state. Although the mechanism for this difference in response sizes in microbial communities within different body habitats is unknown, our findings suggest that oral microbiota might offer advantages in providing biomarkers for oral diseases (or even systematic diseases; Koren *et al.*, 2011).

Conflict of Interest

The authors declare no conflict of interest.

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