Influences of pH and Iron Concentration on the Salivary Microbiome in Individual Humans with and without Caries

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ABSTRACT This study aimed to identify the differences in the oral microbial communities in saliva from patients with and without caries by performing sequencing with the Illumina MiSeq platform, as well as to further assess their relationships with environmental factors (salivary pH and iron concentration). Forty-three volunteers were selected, including 21 subjects with and 22 without caries, from one village in Gansu, China. Based on 966,255 trimmed sequences and clustering at the 97% similarity level, 1,303 species-level operational taxonomic units were generated. The sequencing data for the two groups revealed that (i) particular distribution patterns (synergistic effects or competition) existed in the subjects with and without caries at both the genus and species levels and (ii) both the salivary pH and iron concentration had significant influences on the microbial community structure.

IMPORTANCE The significant influences of the oral environment observed in this study increase the current understanding of the salivary microbiome in caries. These results will be useful for expanding research directions and for improving disease diagnosis, prognosis, and therapy.

KEYWORDS caries, iron, microbial communities, pH, saliva

Dental caries represent a chronic infectious disease with the highest incidence among human oral diseases and a wide distribution (1). In most developed and developing countries, caries are a serious health problem that affect a large proportion of children and adults (2). Therefore, the early diagnosis and prevention of dental caries is important in the 21st century (3).

The oral microbial community structure, rather than a single bacterial species, has been reported to have a stronger influence on oral health, including caries. The structure of the oral microbial community, which includes a range of structural and functional configurations (4), changes in the abundance of certain taxa (5, 6), and the cooccurrence of certain microbes (7), is closely related to caries. Moreover, relationships exist between the microbial communities and many factors in the oral environment (8, 9). Several potential factors have been reported to have significant relationships with caries-associated bacteria, as well as with microbial homeostasis (9–12). For example, intraoral pH has been reported to have a strong effect on the structure of microbial communities, especially for partial dentin caries-associated microbiota, such as some *Lactobacillus* species (12–14). Alternatively, salivary iron, an important elemental metal in saliva, could provide essential nourishment for oral bacterial species (15), and it has been shown to modulate the salivary microbial profile (16). However, the intraoral pH varies (14), and related studies of iron have been mainly based on *in vitro* experiments (16). Moreover, there is no report of the influence of iron on the microbial community

structure in human saliva in patients with and without caries. Thus, more investigations are necessary to determine the clinical significance of the two factors.

In this study, 43 salivary microbial communities were sequenced using the Illumina MiSeq platform. To minimize the effects of the subjects' living environments on the oral microbial communities (9, 17), volunteers with similar living environments and habits were selected from Meipo Village in Jishi Shan, Gansu, China. The influences of oral environmental factors, such as pH and iron concentration, on the bacterial community structure were investigated to determine the role of the microbiome in caries. In the present study, an influence of iron on the microbial community structure in human saliva from patients with and without caries was detected, and salivary pH was synchronously analyzed. The results of this study could increase the current understanding of the correlations between the oral microbiome and caries; this information will be useful for the rapid diagnosis of diseases, for predicting patient prognosis, and for monitoring the targetability and efficacy of therapy for caries.

RESULTS

Similar bacterial diversities in salivary samples from patients with and without caries. After parallel sequencing of the 43 salivary samples, a total of 966,255 trimmed sequences were obtained, with 13,749 normalized reads per microbiome. According to clustering at the 97% similarity level, 1,303 species-level operational taxonomic units (OTUs) were generated, of which 95.2% were shared by the subjects with and without caries. α-Diversity based on four indices did not significantly differ between the subjects with and without caries ($P > 0.05$). A β-diversity comparison indicated that the salivary samples from the patients with and without caries exhibited similar bacterial community structures (see Fig. S1 in the supplemental material).

The OTUs were assigned to 12 phyla, and more than 99% belonged to six phyla (>1% relative abundance), including *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Fusobacteria*, *Actinobacteria*, and *Spirochaetes* (Fig. 1a). A total of 21 classes, 35 orders, 72 families, 145 genera, and 415 species were identified, of which only 9, 11, 15, 17, and 20 members, respectively, were predominant (>1% relative abundance) at each taxonomic level (Fig. 1 and Fig. S2). Within the main taxa (>1% relative abundance), the non-caries-associated taxa based on differential distribution ($P < 0.05$) included the phylum *Bacteroidetes* (Fig. 1a), the class *Bacteroides* (Fig. S2a), the order *Bacteroidales* (Fig. S2b), the genus *Porphyromonas* (Fig. 1b), and the species *Fusobacterium periodonticum* oral taxon 201 (4.31% and 2.31% for the H and C groups, respectively) (Fig. 1c), while the family *Dietziaceae* (Fig. S2c), the genera *Dietzia* and *Selenomonas* (Fig. 1b), and the species *Actinomyces* sp. strain oral taxon 180 (0.09% and 1.07% for the H and C groups, respectively) (Fig. 1c) were associated with caries. Another 53 caries-associated minor taxa (<1% relative abundance), including one class, four orders, 11 families, 18 genera, and 19 species, and 10 non-caries-associated species were also found (Fig. 2a and b). The relative abundance and prevalence of each non-caries- and caries-associated species are further shown in Fig. 2b. Among these species, for example, *Olsenella profusa* oral taxon 806 was detected only in healthy individuals at a low relative abundance (0.003%) but with a prevalence of over 31%. In addition, we compared our results with those of previous studies comparing individuals with and without caries, and the overlapping genera and species are listed in Table S2; discrepancies among the results were due to differences in the sample types, sampling positions, and methods used.

Different cooccurrence patterns in subjects with and without caries. Schoener's cooccurrence index (abundance-based) was calculated for each pair of genera, and heatmaps were generated to separately assess the distribution patterns (Fig. 3). In each group, all genera were gathered into two clusters from the root of the dendrogram based on the similarities of their cooccurrence probabilities, but the assignments in the subcluster were more unbalanced in the caries group than those in the healthy group (Fig. 3a and b). In addition, higher cooccurrence probabilities (Schoener's index of >0.5) were more frequently observed in the subjects with caries than in the healthy subjects
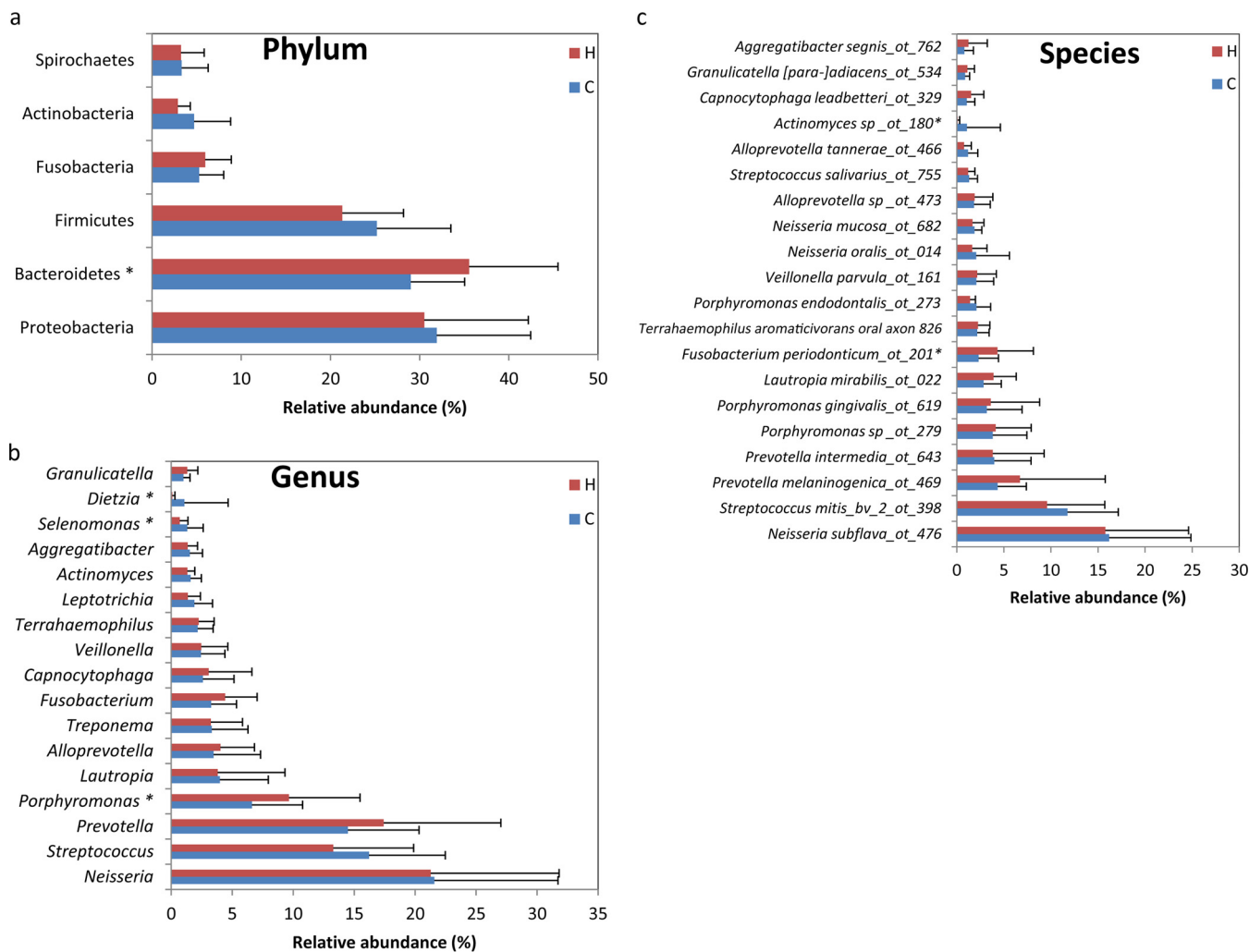
FIG 1 Comparison of bacterial taxonomy (>1% relative abundance) of samples from individuals with (C) and without (H) caries at the phylum (a), genus (b), and species (c) levels. *, $P < 0.05$.

(Fig. 3c). For example, substantial cooccurrence probabilities (>0.8) were only detected between *Microbacterium* and *Agrobacterium* and between *Eggerthella* and *Achromobacter*, whereas a total of 54 pairs, covering 29 genera, had cooccurrence probabilities of greater than 0.8 in the individuals with caries (Fig. 3d). These data indicated that varied distribution patterns existed in the subjects with and without caries.

However, at the species level, similar distribution patterns were detected in the two groups (Fig. S3a), although the genera harboring higher cooccurrence probabilities (Schoener's index of >0.8) were still more prevalent in the caries group than in the healthy group (Fig. S3b).

Salivary pH has a negative relationship with iron concentration. Both the pH and iron concentration significantly differed between the subjects with and without caries ($P < 0.001$) (Fig. 4a and b). A significant negative relationship between the salivary pH and iron concentration was observed in the 43 individuals (Fig. 4c). However, only the salivary pH was significantly correlated with the decayed, missing, and filled teeth (DMFT) index (Fig. 4d).

Influence of important variables on bacterial community structure. The salivary pH, oral iron concentration, and DMFT index significantly influenced the bacterial community structure at the genus and species levels ($P < 0.01$) (Fig. 5). The redundancy analysis (RDA) plots showed that the caries- and non-caries-associated genera could be divided into two groups: those highly related to one variable (e.g., *Oribacterium* with
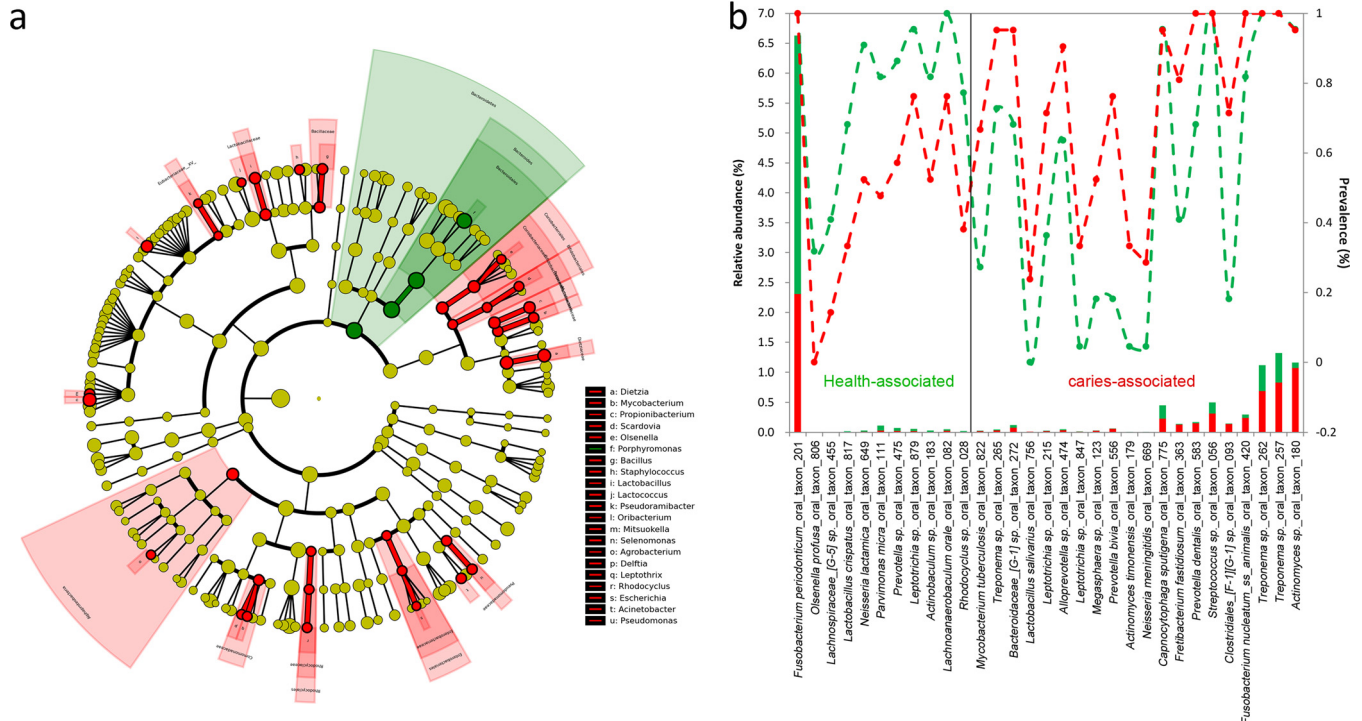
FIG 2 Non-caries- and caries-associated genera (a) and species (b), as determined based on the LEfSe method. The nonparametric factorial Kruskal-Wallis rank sum test was used to detect the taxa with significant differential relative abundance between the healthy group and the caries group at a significance level of 0.05. The relative abundance (the histograms) and prevalence (the dotted lines) of each species are also shown in panel b. Green and red indicate data from healthy and caries groups, respectively.

salivary pH), or to more than one variable, and those clustered near the DMFT arrow (Fig. 5). As shown in the Venn diagram in Fig. 5, in addition to the genus *Oribacterium*, briefly, a cluster of 12 caries-associated genera was correlated with the DMFT. Three other caries-associated genera, including *Olsenella*, *Scardovia*, and *Pseudoramibacter*, showed high correlations with pH and iron, and the others, including four caries- and one non-caries-associated genera, were highly related to all three variables. However, the correlations between the factors and species were more complex (Fig. S4), and the species exhibiting strong correlations are further listed in Table 1.

Moreover, at the species level, the salivary pH, iron concentration, and DMFT index also exhibited substantial effects. For example, *Ochrobactrum anthropi* oral taxon 544 and TM7 [G-4] sp. strain oral taxon 355 showed strong negative and positive correlations, respectively, with the DMFT index. Alternatively, *Prevotella multisaccharivorax* oral taxon 794 and *Enterococcus italicus* oral taxon 803 exhibited strong negative correlations with the salivary pH and iron concentration, respectively. In addition, *Lactobacillus salivarius* oral taxon 756 and *Leptotrichia* sp. strain oral taxon 847 overlapped with the caries-associated species based on differential distribution analysis.

DISCUSSION

In the present study, we examined the similarities and differences in salivary bacterial communities between individuals with and without caries using high-throughput sequencing. Because it is relatively stable, easy, and inexpensive to acquire, saliva is an almost ideal biological secretion for studies of the oral microbial profile in health and disease (18, 19). However, it also should be noted that saliva might not completely represent the bacterial diversity at the disease site, which should be addressed in further studies and clinical applications (7, 20). To obtain a more comprehensive understanding, we included recent reports on caries-associated microbial populations (see Table S2 in the supplemental material). Eighty percent of these reports concerned children (0.6 to 8 years), early young adults (8 to 16 years), or young adults
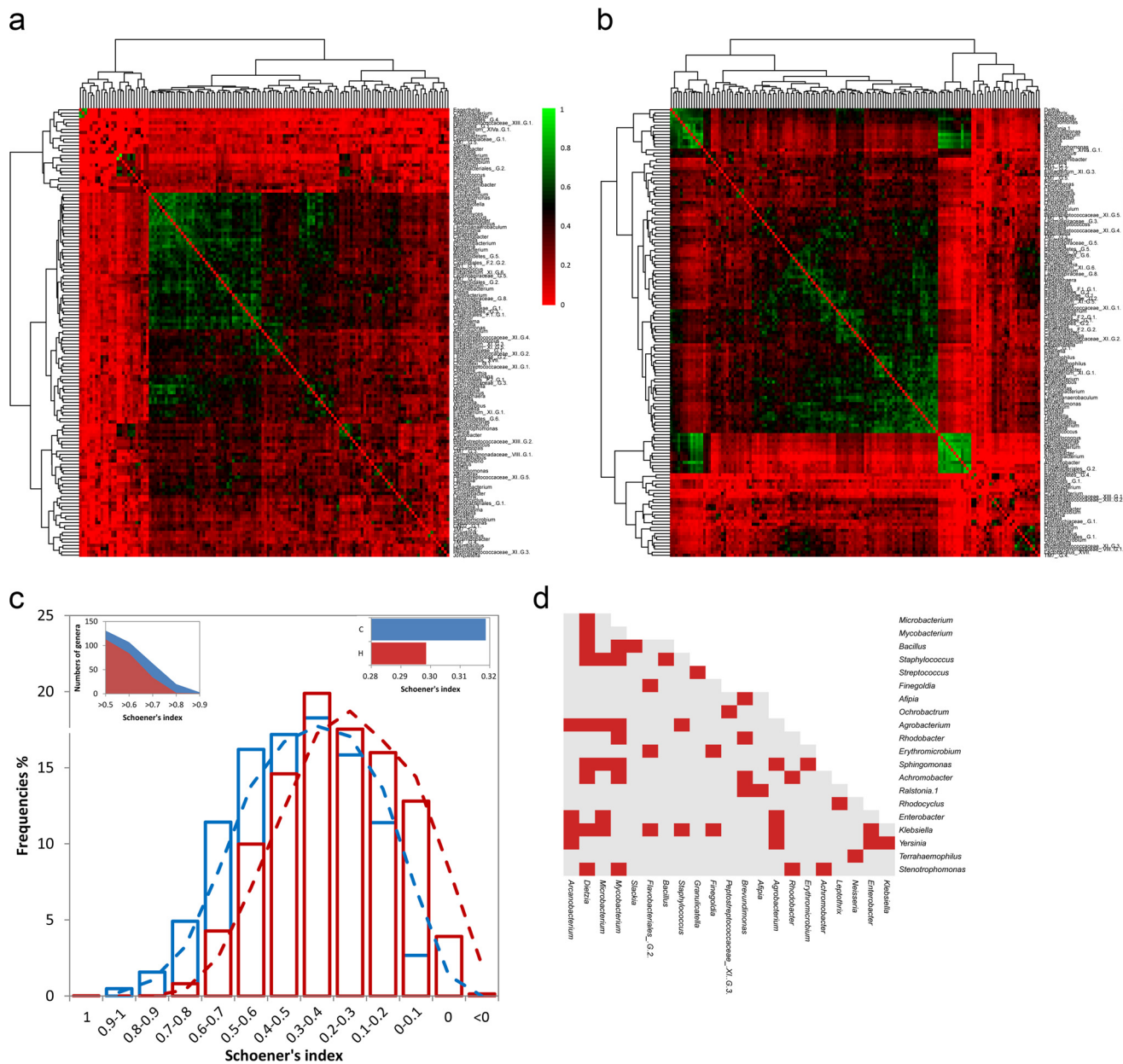
FIG 3 Heatmaps displaying genus distribution patterns for the healthy (a) and caries (b) groups. Increasing values are translated into colors from red to green. Trees were clustered based on the similarity of the Schoener's values. (c) The cooccurrence probabilities based on the Schoener's index were divided into 13 intervals, including <0, 0, 0 to 0.1, 0.1 to 0.2, 0.2 to 0.3, 0.3 to 0.4, 0.4 to 0.5, 0.5 to 0.6, 0.6 to 0.7, 0.7 to 0.8, 0.8 to 0.9, 0.9 to 1, and 1. The frequency of the Schoener's index falling into each interval was calculated in both the caries group and the healthy group. The number of involved genera at the different intervals and a global comparison of the Schoener's index between the two groups (using a t test) are also shown within the plot. (d) All pairs with Schoener's index greater than 0.8.

(18 to 22 years). Neither the bacterial diversity nor the caries-associated taxa identified have been consistent among studies, which may be related to differences in sampling and/or analysis methodologies, as well as differences in environmental factors (9). Therefore, further analyses at different sites are necessary to verify the identities of the critical taxa and to confirm whether these taxa are consistent from one population to the next. Furthermore, we should also focus on the entire microbial community structure in addition to the characteristic members (21). In our study, the microbiomes from 43 adults were investigated, and different distribution patterns were observed in the subjects with and without caries at the genus level. We previously reported similar results based on a higher Schoener's cooccurrence index in the caries group than in the
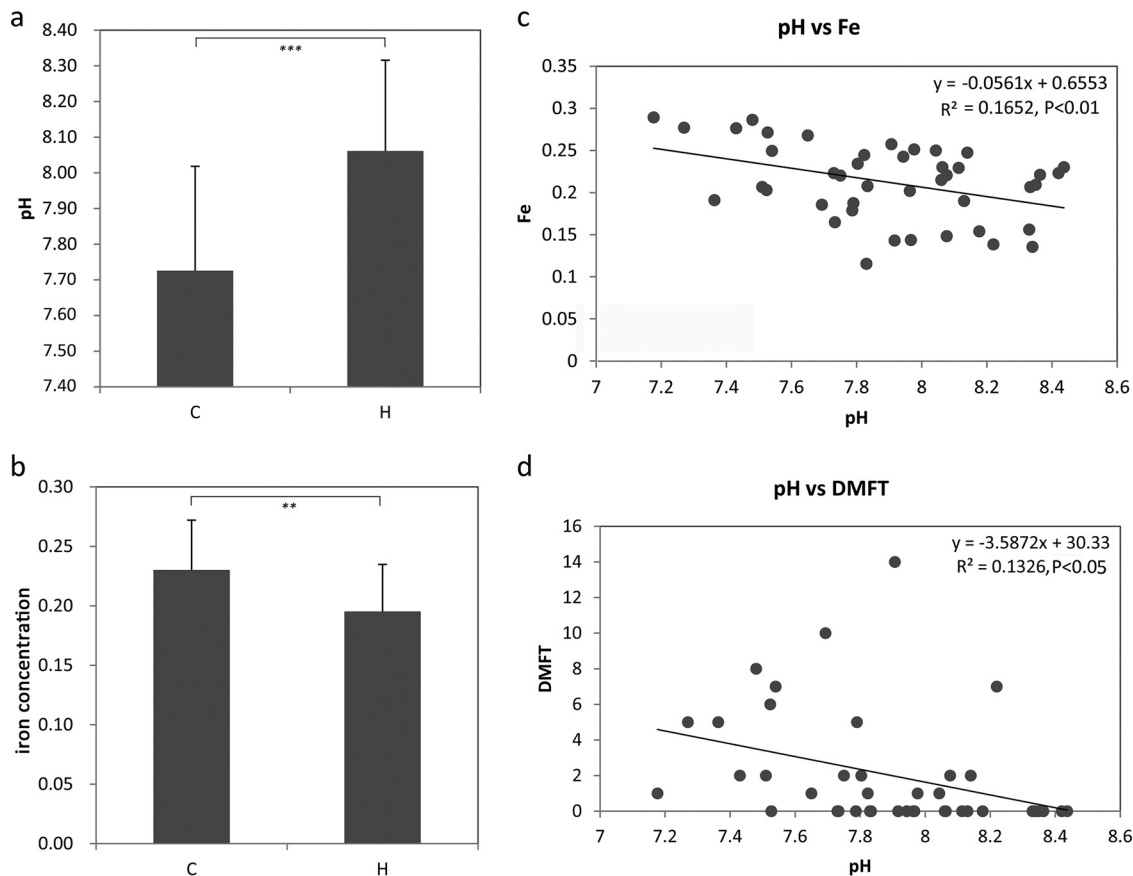
FIG 4 Salivary pH and iron concentration. Significant differences in the salivary pH (a) and iron concentration (b) between samples from individuals with (C) and without (H) caries. ***, $P < 0.001$; **, $P < 0.01$. (c) Significant correlations between the salivary pH and iron concentration. (d) Significant correlations between the salivary pH and DMFT index.

healthy group (7). Simultaneously, given the higher frequencies of Schoener's index in the subjects with caries observed in the present study, we propose that bacterial assemblages exhibit an aggregation structure (i.e., high cooccurrence) more often in caries, suggesting that mutualistic or syntrophic and competitive interactions dominate (22, 23) in the caries and healthy groups, respectively. Of course, further experimental evidence is needed.

Furthermore, pairs with higher cooccurrence probabilities were profiled at both the genus and species level. However, the distribution patterns at the species level were similar in the caries and healthy groups. Different distribution patterns at different taxonomic levels were also previously reported in the oral cavity of healthy individuals (24), and we believe that the interactions of species are complex and that a deeper investigation of microorganisms at the species level is required.

Relationships of the bacterial profiles with pH and iron concentration. As expected, the structure of the salivary bacterial community was significantly influenced by the pH and iron concentration ($P < 0.01$). However, in the RDA plots at both the genus and species levels, obtuse angles between the pH and iron concentration were observed, suggesting opposite trends of the two factors. Most of the non-caries- and caries-associated taxa were strongly correlated with these variables, suggesting an important relationship between these characteristic taxa and the environment. Low pH has been proposed to cause a shift in acid-tolerant and acid-producing bacterial consortia, such as those observed in our study, namely, *Lactobacillus vaginalis* and *Streptococcus mitis* (25, 26), which favors the formation of caries lesions (11, 27). Additionally, in the current study, species strongly correlated with high iron included caries-associated bacteria such as *Propionibacterium acidifaciens* (28), acid-tolerant

FIG 5 Redundancy analysis (RDA) of the significant influences of the salivary pH, iron concentration, and DMFT index on the bacterial community structure at the genus level. Colors indicate the non-caries-associated (underlined) and caries-associated genera. These distinct genera with high correlations with one or more variables are explained by the Venn diagram inserted in the plot: yellow, blue, and red indicate genera with high correlations (>0.6) with iron concentration, pH, and DMFT, respectively.

strains such as *Streptococcus constellatus* (26) and *Prevotella oulorum* (11), as well as *Streptococcus oligofermentans*, which has been reported to inhibit the overgrowth of cariogenic pathogens (29). Therefore, we hypothesize that both a low pH and high iron concentration influence salivary species, which further increases the risk of caries.

TABLE 1 Details for species with high correlations with the three variables[f]

Variable and no.[a]	Phylum	Species	HOT[b]
Positive			
pH			
S40	*Bacteroidetes*	*Capnocytophaga* sp.	332
S269[e]	*Bacteroidetes*	*Prevotella multisaccharivorax*	794
S227	*Bacteroidetes*	*Prevotella oralis*	705
S45	*Bacteroidetes*	*Alloprevotella* sp.	912
S382[e]	*Firmicutes*	*Streptococcus sobrinus*	768
S158	*Fusobacteria*	*Leptotrichia buccalis*	563
S1	*Proteobacteria*	*Neisseria subflava*	476
S154	*Proteobacteria*	*Cardiobacterium hominis*	633
S237[c]	*Proteobacteria*	*Neisseria lactamica*	649
S344	*Spirochaetes*	*Treponema* sp.	227
S306	*Tenericutes*	*Mycoplasma faucium*	606
Iron			
S43	*Bacteroidetes*	*Prevotella* sp.	299
S116	*Bacteroidetes*	*Prevotella veroralis*	572
S194	*Bacteroidetes*	*Capnocytophaga* sp.	863
S330[e]	*Firmicutes*	*Lachnoanaerobaculum* sp.	496
S207	*Firmicutes*	*Mitsuokella* sp.	521
S218	*Firmicutes*	*Moryella* sp.	419
S46	*Firmicutes*	*Streptococcus constellatus*	576
S60[d]	*Firmicutes*	*Streptococcus* sp.	56
S351	*Proteobacteria*	*Desulfovibrio* sp.	40
S38	*Spirochaetes*	*Treponema amylovorum*	541
S123[d]	*Synergistetes*	*Fretibacterium fastidiosum*	363
DMFT			
S183	*Actinobacteria*	*Atopobium rimae*	750
S310[e]	*Bacteroidetes*	*Capnocytophaga* sp.	902
S125	*Bacteroidetes*	*Prevotella shahii*	795
S234[d]	*Firmicutes*	*Megasphaera* sp.	123
S181	*Firmicutes*	*Lachnospiraceae* [G-2] sp.	88
S120[d]	*Firmicutes*	*Clostridiales* [F-1][G-1] sp.	93
S109	*Firmicutes*	*Veillonellaceae* [G-1] sp.	155
S54	*Spirochaetes*	*Treponema denticola*	584
S352[e]	TM7	TM7 [G-4] sp.	355
Negative			
pH			
S141	*Bacteroidetes*	*Bacteroidetes* [G-6] sp.	516
S98	*Bacteroidetes*	*Bacteroidetes* [G-5] sp.	505
S14	*Bacteroidetes*	*Porphyromonas endodontalis*	273
S256	*Bacteroidetes*	*Bacteroidetes* [G-5] sp.	511
S51	*Bacteroidetes*	*Prevotella* sp.	526
S108	*Firmicutes*	*Lachnospiraceae* [G-3] sp.	100
S412	*Firmicutes*	*Enterococcus saccharolyticus*	802
S189	*Firmicutes*	*Peptostreptococcaceae* [XI][G-4] sp.	369
S149	*Firmicutes*	*Lactobacillus vaginalis*	51
S387[e]	*Firmicutes*	*Enterococcus durans*	880
S241	*Fusobacteria*	*Leptotrichia* sp.	218
S203	*Fusobacteria*	*Fusobacterium* sp.	370
S122	*Fusobacteria*	*Leptotrichiaceae* [G-1] sp.	210
S128	*Proteobacteria*	*Haemophilus parainfluenzae*	718
S71	*Spirochaetes*	*Treponema* sp.	235
S200	*Spirochaetes*	*Treponema* sp.	226
Iron			
S408	*Actinobacteria*	*Bifidobacterium dentium*	588
S291	*Actinobacteria*	*Olsenella* sp.	807
S69	*Actinobacteria*	*Corynebacterium matruchotii*	666
S295	*Actinobacteria*	*Actinomyces* sp.	897
S276	*Bacteroidetes*	*Porphyromonas* sp.	275
S68	*Bacteroidetes*	*Flavobacteriales* [G-1] sp.	318
S40	*Bacteroidetes*	*Capnocytophaga* sp.	332
S36	*Bacteroidetes*	*Prevotella denticola*	291
S260	*Firmicutes*	*Enterococcus italicus*	803

(Continued on following page)

TABLE 1 (Continued)

Variable and no.[a]	Phylum	Species	HOT[b]
S198	*Firmicutes*	*Peptostreptococcaceae* [XI][G-5] sp.	493
S375	*Proteobacteria*	*Ralstonia* sp.	27
S1	*Proteobacteria*	*Neisseria subflava*	476

[a]No. indicates the number of the species in the RDA plot (see Fig. S4); boldface font indicates a correlation of >0.996 ($\pm 5°$).
[b]HOT indicates the human oral taxon ID in the Human Oral Microbiome Database (http://www.homd.org/index.php).
[c]Non-caries-associated species.
[d]Caries-associated species.
[e]Low-abundance species with a community-wide impact.
[f]Correlation value of >0.985 ($\pm 10°$) (Fig. 5b).

However, higher pH and lower iron may not necessarily be safe for oral health. In our pH-positive (higher pH) profiles, *Streptococcus sobrinus*, previously reported as an etiologic agent of dental caries (30), was also observed, although with a low relative abundance (average of approximately 0.001%) and low prevalence (<20% in total). Similarly, the lower iron profile also included the potentially caries-associated pathogenic species *Bifidobacterium dentium* (31), although with low relative abundance (average of approximately 0.001%) and low prevalence (<20% in total). In addition, higher pH and lower iron profiles harbored several periodontitis-assorted species, including *Aggregatibacter actinomycetemcomitans*, *Dialister pneumosintes* (32), *Prevotella oralis* (33), *Capnocytophaga sputigena*, *Cardiobacterium hominis* (34), and *Treponema lecithinolyticum* (35). All of these data suggest that either higher or lower pH or iron creates potentially pathogenic conditions, and thus a single product for daily oral care probably should be avoided; the ranges of pH and iron concentration necessary for maintaining a healthy oral environment require further investigation.

Relationships among the DMFT index, pH, and iron concentration based on correlations with salivary microbiomes. The DMFT index, an index of the dental caries burden, was also significantly correlated with the bacterial community structure ($P < 0.01$). In the RDA plot (Fig. 5), the clustered caries-associated genera exhibited closer relationships with the DMFT index than with the pH or iron concentration, indicating that their cooccurrence is more strongly influenced by the DMFT index. Additionally, a higher DMFT index was consistently observed along with a reduced pH and increased iron concentration (Fig. 5 and Fig. S4). These findings indicate a potential clinical method for assessment of lesion acidity based on intraoral pH measurement (12); however, they contradict the cariostatic properties of iron (15). It is well accepted that the development of dental caries involves the dissolution of the tooth structure by acid produced by oral bacteria as a result of the fermentation of dietary carbohydrates, and aciduric or acid-producing species are the major cariogenic species (36). A certain amount of iron could reduce enamel demineralization (15, 37–41) through the formation of an acid-resistant coating on the enamel surface (42). Although the studies mentioned above evaluated the effects of iron(II) supplements (often ferrous sulfate) coupled with sugar or acidic solutions, neither the actual iron concentration nor the pH in the oral cavity has been reported. In the present study, the iron concentration representing the entire iron element in saliva was detected, including iron(II) and iron(III). Iron(III) complex addition has been reported to have no cariostatic effect (43). Therefore, different valences of iron should be considered in the future. Additionally, studies based on *in vitro* experiments have reported that iron compounds can form on the enamel surface, which indicates that it is also essential to develop methods to increase the percentage of change in superficial hardness and prevent remineralization in addition to other adverse effects, such as toxicity and tooth staining (15, 38). The reduced capacity of artificial saliva to promote remineralization was more substantial under higher iron concentrations (15). In our study, the iron concentration showed strong relationships with several of the caries-associated species mentioned above, which may have disrupted the balance of the bacterial community structure. Given the

significant correlation of the iron concentration with the pH, which was not observed with the DMFT index, we suspect that the data for the pH and iron concentration represent the real-time conditions of the collected samples, whereas the DMFT index represents activities that mainly occurred in the past. In addition, a lower pH could enhance the solubility of iron and increase the availability of iron for oral microbial growth (44), which might result in a microbiota with a potential clinical impact on the formation of caries (16). Therefore, a possible explanation for the lack of a significant correlation between the iron concentration and the DMFT index is that the low solubility of iron (16) under aqueous and neutral pH conditions (45) limited the consistent increase in the iron concentration along with the DMFT index. Alternatively, individuals who develop caries may harbor a higher prevalence of certain acid-producing or acid-tolerant species that prefer higher salivary iron concentrations, which is consistent with the higher iron profile described above. Considering the importance and significant influences of iron and pH on the salivary microbial community in the present and previous studies (16, 19), the pH-iron correlation might have, at least in part, contributed to the oral microorganisms. Consequently, we propose that long-term investigation of the conversion of the oral microbiota and its correlation with pH in the presence of iron should be conducted before iron-containing products can be recommended.

MATERIALS AND METHODS

Subjects and specimen collection. Volunteers were selected from Meipo Village, Jishishan Autonomous County, Gansu Province, China, in September 2014. Individuals were excluded from this study for the following reasons: they had taken antibiotics during the previous 3 months or had received systemic periodontal treatment in the preceding year; they had a systemic disease or immune suppression; and/or they were pregnant or smokers. All subjects did not eat and had undergone a 12-h period without oral care prior to initiation of the study. A total of 21 subjects with inactive caries and 22 healthy subjects (24 to 56 years old) were recruited and classified into C and H groups (see Table S1 in the supplemental material), respectively. As this study involved experiments on humans, the study protocols conformed to the guidelines of the Declaration of Helsinki. After obtaining informed written consent and with approval of the Ethics Committee of Northwest University for Nationalities, 1 ml of unstimulated saliva was collected from each subject the next morning.

pH and iron concentration measurements and DNA extraction. One milliliter of unstimulated saliva was mixed with 20 ml normal saline for use in further experiments. All saline-saliva samples were centrifuged at 12,000 rpm for 5 min. Ten milliliters of supernatant was used for measurement of the pH with a calibrated FE20 FiveEasy Plus pH meter (Mettler-Toledo, Schwerzenbach, Switzerland). Two milliliters of supernatant was used for measurement of the iron concentration with a Thermo elemental atomic absorption spectrometer (Thermo Scientific, East Lyme, CT). Briefly, standards containing known concentrations of iron were prepared by serial dilutions. The standards and samples then were dried at 80°C and 130°C for 20 s and 10 s, respectively, ashed, and atomized at 1,200°C and 2,100°C for 10 s and 5 s, respectively. Spectrophotometric measurements of the samples were performed at a wavelength of 243.8 nm. A calibration curve was generated using the measurements for the standards and was then used to quantify the iron levels in the samples and blank (normal saline only); the difference between the sample and blank was the final value. The samples used for measurement of the pH and iron concentration were analyzed in triplicate. The entire pellet was used to extract salivary DNA, and salivary DNA extraction was performed using a Qiagen Stool minikit (Qiagen, Valencia, CA) as previously described (7).

Highly parallel DNA sequencing. The 16S rRNA gene V4-V5 region was amplified using the following primers: 5′-GTGCCAGCMGCCGCGG-3′ and 5′-CCGTCAATTCMTTTRAGTTT-3′. PCR amplification was performed with an Eppendorf Mastercycler EP gradient thermal cycler (Eppendorf, Hauppauge, NY) in a total volume of 25 μl containing 9 μl of sterilized water, 5 μl of 5× PCR GC high enhancer, 5 μl of 5× PCR buffer, 2 μl of 2.5 mM deoxynucleoside triphosphates (dNTPs), 2 μl of 200 ng/μl template DNA, 0.25 μl of 5 U/μl TaKaRa polymerase, and 1 μl of each primer (10 μM). The thermal cycling conditions were initial denaturation at 98°C for 5 min, followed by 27 cycles at 98°C for 30 s, 50°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 5 min. The PCR products (3 μl) were detected on an agarose gel (2.0%). Each PCR product was tagged with an index sequence at the 5′ end of the forward primer. Purified PCR amplicons were used to construct paired-end DNA libraries, which were then run on the Illumina MiSeq (250-bp paired-end reads) platform.

Quantitative Insights Into Microbial Ecology (QIIME) toolkit v.1.7.0 was used to trim the raw sequences (46). Reads that were shorter than 150 bp, those that contained any ambiguous bases, or those that contained a homopolymer of longer than 8 bp were removed, and chimeric sequences were identified and removed using the UCHIME tool of the mothur software package (v.1.31.2) (47, 48). All of the trimmed sequences were normalized to the same sequencing depth using mothur. The operational taxonomic units (OTUs) were clustered at 97% similarity using the uclust tool of QIIME software.

Representative sequences for each OTU were searched against the Human Oral Microbiome Database (HOMD; http://www.homd.org/) (49).

Statistical analysis. The Shannon, Simpson, Chao, and ACE indices (50, 51) were calculated for α-diversity measurement using mothur. Weighted UniFrac distance matrices (52) were calculated using QIIME. To estimate β-diversity, principal coordinate analysis (PCoA) was performed using the R package vegan. The linear discriminant analysis effect size (LEfSe) method (53–55) was used to compare the bacterial community structures between the samples from the patients with and without caries, as in previous reports (7). Schoener's index (abundance-based) (56) was computed to measure the cooccurrence probability for each pair of genera or species using the R spaa package, which was then used to construct heatmaps to evaluate the different distribution patterns in the two groups using the ggplots package in R, as described previously (7). Redundancy analysis (RDA) was performed using the vegan package in R to test the influences of the salivary pH, iron concentration, and DMFT index on the microbial community structure at the species level. In the RDA plot of species, the arrows pointing to the factors were rotated 5 and 10 degrees positively and negatively, respectively. Species that fell in this interval were considered to have strong correlations ($\cos 5° \approx 0.996$ and $\cos 10° \approx 0.985$). In addition, the distance to the origin was calculated, and only values of >0.5 are shown in Table 1.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02412-16.

TEXT S1, PDF file, 1.4 MB.

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