

High-throughput sequencing analyses of oral microbial diversity in healthy people and patients with dental caries and periodontal disease

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Received April 2, 2016; Accepted March 7, 2017

DOI: 10.3892/mmr.2017.6593

Abstract. Recurrence of oral diseases caused by antibiotics has brought about an urgent requirement to explore the oral microbial diversity in the human oral cavity. In the present study, the high-throughput sequencing method was adopted to compare the microbial diversity of healthy people and oral patients and sequence analysis was performed by UPARSE software package. The Venn results indicated that a mean of 315 operational taxonomic units (OTUs) was obtained, and 73, 64, 53, 19 and 18 common OTUs belonging to *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria* and *Fusobacteria*, respectively, were identified in healthy people. Moreover, the reduction of *Firmicutes* and the increase of *Proteobacteria* in the children group, and the increase of *Firmicutes* and the reduction of *Proteobacteria* in the youth and adult groups, indicated that the age bracket and oral disease had largely influenced the tooth development and microbial development in the oral cavity. In addition, the traditional 'pathogenic bacteria' of *Firmicutes*, *Proteobacteria* and *Bacteroidetes* (accounted for >95% of the total sequencing number in each group) indicated that the 'harmful' bacteria may exert beneficial effects on oral health. Therefore, the data will provide certain clues for curing some oral diseases by the

strategy of adjusting the disturbed microbial compositions in oral disease to healthy level.

Introduction

The oral cavity contains a large number of sites, which are suitable for the growth and assemblage of various microorganisms (1). To date, ~700 phylotypes have been identified in the oral cavity and half of these microbes exist in any individual during their lifetime (2). These microorganisms, which are predominantly composed of bacteria, may prevent oral colonization from exogenous organisms, indicating that they are indispensable to health (3). In addition, many normal oral inhabitants are associated with some oral diseases including dental caries, gingivitis, periodontitis, candidiasis, endodontic infections, orthodontic infections and peri-implantitis (2,4-7). Therefore, it is crucial to investigate the actual microbe compositions in healthy people and dental patients for understanding the relationship between bacteria and various oral diseases.

In general, bacterial infections within the oral cavity are polymicrobial in nature, and it is quite unusual for any oral disease caused by a single species (8). However, medical microbiologists have relied on culture techniques to elucidate the complexity of infections for decades, and these culture methods can only be used for identifying the 'culturable' bacteria that are growing relatively quickly and easily in laboratory media (9,10), and it is technically difficult to separate and identify >3-6 species because of their various requirements of nutrition, pH, temperature and oxygen, and the interspecies competition and different concentrations of bacteria on the culture plate also increase the difficulties of screening (11,12). To determine the roles of bacteria in oral diseases, it is necessary to define the full panoply of organisms within the human mouth, and high-throughput sequencing methods may be a useful method for alleviating this problem since it can detect almost all the DNA signatures of microorganisms within a specific environment, even those present in low numbers or in a dormant metabolic state (13-15).

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Key words: high-throughput sequencing analyses, dental caries, periodontal disease, microbial diversity

As is already known, dental caries is a destructive condition of the dental hard tissues that can progress to inflammation and death of vital pulp tissue, with eventual spread of infection to the periapical area of the tooth and beyond (16). Conversely, periodontal diseases can involve in both the soft and hard tissues and are the most common inflammatory destructive conditions that affect humans (8). To eliminate the overestimation of the importance of species that are easily cultured and the underestimation of the fastidious organisms that may be highly prevalent and important in dental caries and periodontal diseases, high-throughput sequencing analyses were used in the present study.

Materials and methods

Ethical statement. The study was approved by the Ethical Committee of the Stomatological Hospital of Nanchang University and all participants provided written informed consent.

Patient selections and sampling. The patients only with typical dental caries and periodontal diseases were selected from the Stomatological Hospital of Nanchang University (Nanchang, China). Patients with dental caries were recorded if either cumulative caries (dmft/DMFT) or initial caries score was >0 or the *Streptococcus mucans* count was ≥ 105 CFU/ml (17), and periodontal disease was defined as two or more teeth with clinical attachment loss (CAL) or ≥ 4 mm (18). Then, the patients were divided into seven groups: Children-Control group (C.C, n=5, 6-18 years old), Children-Dental caries group (C.DC, n=8, 6-18 years old); Youth-Control group (Y.C, n=8, 18-35 years old), Youth-Dental caries group (Y.DC, n=8, 18-35 years old); Adult-Control group (A.C, n=7, 35-60 years old), Adult-Dental caries group (A.DC, n=6, 35-60 years old), and Adult-Periodontitis group (A.P, n=8, 35-60 years old). All of the subjects were Chinese, and those patients who were pregnant, lactating or had other systemic conditions were excluded. In addition, none of the subjects had received systemic antibiotics or periodontal therapy in the previous 6 months (19).

Stimulated whole saliva and oropharyngeal samples were collected over a 6-month period from healthy and dental patients. Subjects chewed a 1 g piece of paraffin wax for 1 min, and after swallowing once, they expectorated secreted saliva into a sterile plastic 50 ml tube several times and this was kept frozen at -20°C until processing. The oropharyngeal samples were collected using dry cotton to gently swab the posterior wall of the oropharynx in which the samples were directly suspended in a microtube containing 200 μl lysis buffer [Tris 20 mM, EDTA 2 mM (pH 8), Tween-20 1%, proteinase K (Thermo Fisher Scientific, Inc., Waltham, MA, USA) 400 $\mu\text{g}/\text{ml}$]. Then, saliva samples with the same volume of 2X lysis buffer and the oropharyngeal lysates were mixed in a 1:10 ratio (19,20), and 200 μl mixture of saliva and oropharyngeal lysates from every patient in same group were mixed together for DNA extraction (21).

Extraction of genome DNA and high-throughput sequencing. Genomic DNA of each sample was extracted by a TIANamp Genomic DNA kit (Tiangen Biotech Co., Ltd., Beijing, China)

combined with bead beating, as previously published (22). The extracted genomicDNA was used as the template to amplify the V3region of 16S rRNA genes using primer of 515F/806R with the barcode. PCR reactions, pyrosequencing of the PCR amplicons and quality control of raw data were performed as described previously (23).

Bioinformatics and multivariate statistics. Paired-end reads from the original DNA fragments were merged using FLASH (Fast Length Adjustment of Short Reads to Improve Genome Assemblies; <http://www.cbcb.umd.edu/software/flash>) to merge paired-end reads when at least some of the reads overlap the read generated from the opposite end of the same DNA fragment, and paired-end reads were assigned to each sample according to the unique barcodes.

Then, sequences analysis was performed by UPARSE software package version 7.0.1001 (<http://drive5.com/uparse/>) using the UPARSE-operational taxonomic unit (OTU) and UPARSE-OTUref algorithms. In-house Perl scripts were used to analyze alpha (within samples) and beta (among samples) diversity. Sequences with $\geq 97\%$ similarity were assigned to the same OTUs. Sequence was picked as a representative for each OTU, and the Ribosomal Database Project (RDP; <http://rdp.cme.msu.edu/classifier/classifier.jsp>) classifier was used to annotate taxonomic information for each representative sequence. Cluster analysis was preceded by unweighted UniFrac distance using the Quantitative Insights into Microbial Ecology software package version 1.8.0 (QIIME; <http://qiime.org/index.html>).

Results

Shared genera in healthy people and dental patients. To compare the microbes in healthy people and dental patients, 16S rRNA amplicon sequencing analysis was used to sequence the V4 hypervariable region, and the sequencing data was filtered to get the valid data. All the effective tags of all samples were clustered and those sequences with over 97% similarity were considered as one OTU. In total, 1,321,995 usable raw sequences (8,686 unique sequences) and 2,207 OTUs were obtained from all the samples with an average of 315.3 OTUs per group (Table I). The Chao1 and Shannon indexes were almost saturated and the rarefaction curve of every sample was able to enter the plateau phase (data not shown).

The Venn figure may reflect the difference between all groups. As presented in Fig. 1, there were 305 (C.C), 285 (C.DC), 305 (Y.C), 335 (Y.DC), 288 (A.C), 331 (A.DC), and 358 (A.P) OTUs in each group, and there were 237 common OTUs in the C.C, Y.C and A.C groups (healthy group, Fig. 1D); of which 73, 64, 53, 19 and 18 OTUs belonged to *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria* and *Fusobacteria* respectively (data not shown). The common OTUs in the C.C, Y.C and A.C groups (disease group, Fig. 1E) was 248, of which 79, 64, 54, 19 and 14 OTUs belonged to *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Fusobacteria* and *Actinobacteria* respectively (data not shown). It should be noted that there were only three common OTUs in the C.DC, A.DC and Y.DC groups belonging to *Spirochaetes* (1 OTU) and *Proteobacteria* (2 OTUs), and the unique OTUs in the

Table I. Number of total tags, taxon tags, unclassified tags, unique tags and OTUs from the oral cavity of healthy people and patients with dental caries and periodontal disease by high-throughput sequencing.

Group	Total tags	Taxon tags	Unclassified tags	Unique tags	OTUs
C.C	182,851	181,759	0	1,092	305
C.DC	159,863	158,641	0	1,222	285
Y.C	206,886	205,408	0	1,478	305
Y.DC	151,942	151,027	0	915	335
A.C	188,805	187,700	0	1,105	288
A.DC	168,826	167,602	0	1,224	331
A.P	262,822	261,172	0	1,650	358
Mean	188,856	187,615.6	0	1,241	315
Total	1,321,995	1,313,309	0	8,686	2,207

OTUs, operational taxonomic units.

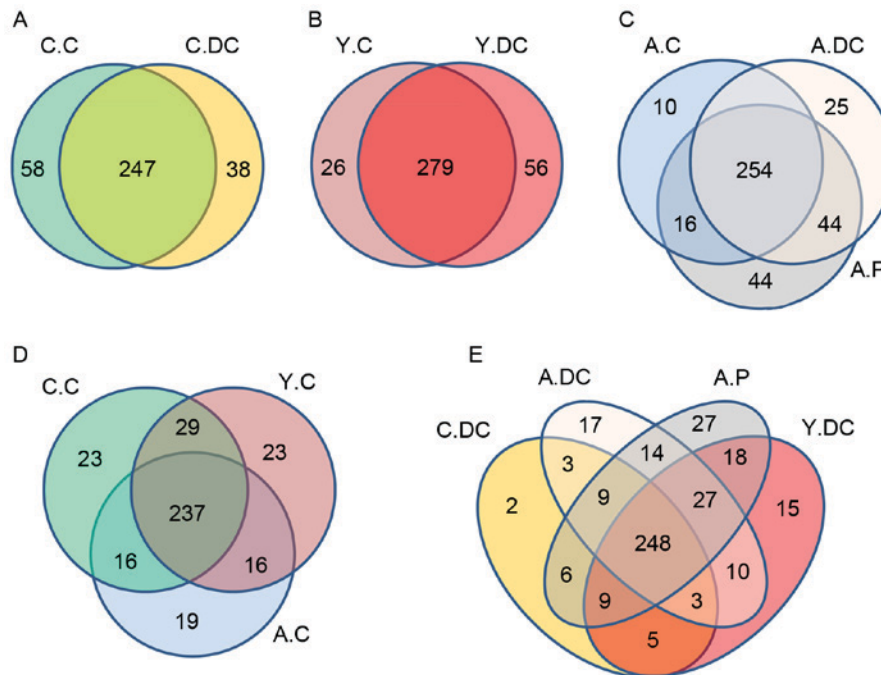


Figure 1. Scalar-Venn representation of the oral microbiota among C.C, C.DC, Y.C, Y.DC, A.C, A.DC and A.P groups. The shared OTUs between (A) C.C and C.DC; (B) Y.C and Y.DC; (C) A.C, A.DC and A.P; (D) C.C, Y.C and A.C; (E) C.DC, A.DC, A.P and Y.DC groups. C.C, Children-Control group; C.DC, Children-Dental caries group; Y.C, Youth-Control group; Y.DC, Youth-Dental caries group; A.C, Adult-Control group; A.DC, Adult-Dental caries group; A.P, Adult-Periodontitis group.

A.P group was 27, of which *Actinobacteria* only occupied 1 OUT, while *Proteobacteria* and *Spirochaetes* occupied 5 and 3 OUTs, respectively.

Compositions and relative abundance of bacterial communities in phylum level. Based on the weighted UniFrac distance in phylum level, data for the top 10 microorganism populations were analyzed using unweighted Pair-group Method with Arithmetic Mean (UPGMA) to check the similarity between different groups (24). As presented in Fig. 2, *Firmicutes*, *Proteobacteria* and *Bacteroidetes* constituted the three commonest dominant phyla and accounted for >95% of the total sequencing number in all groups, of which the percentage

of *Firmicutes* was ~75% in the Children-Control group (C-C, n=5, 6-18 years old), 48% in Youth-Control group (Y-C, N=8, 18~35 years old) and 36% in the Adult-Control group (A-C, n=7, 35-60 years old). The percentage for *Proteobacteria* was 10% in the C.C group, 32% in the Y.C group and 50% in the A.C group (Fig. 2), respectively. Notably, the percentage of *Bacteroidetes* was relatively stable, maintaining a ratio of 10% of the total sequencing number.

Compared with the healthy people, the percentages of *Firmicutes* and *Proteobacteria* were reduced from 75 to 45% in dental caries patients, and from 10 to 39% in the children group (C.C vs. C.DC), whereas the percentage of *Firmicutes* was increased from 48 to 58% and *Proteobacteria* was reduced

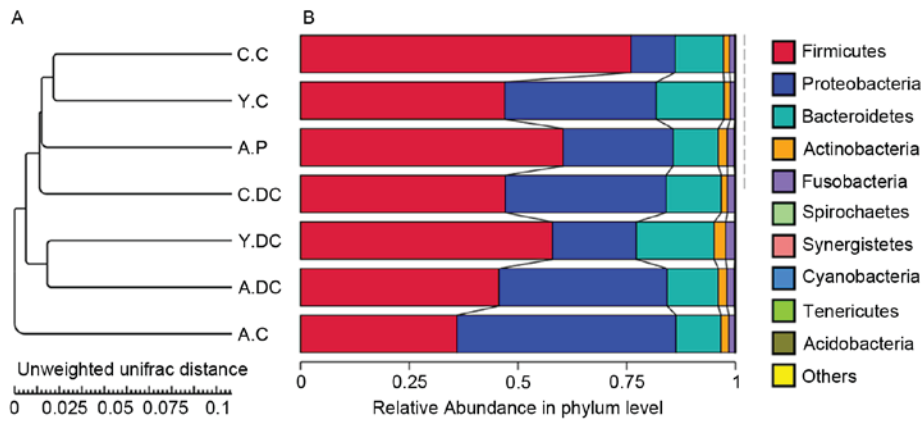


Figure 2. Composition and relative abundance of bacterial communities based 16S rDNA sequences in C.C, C.DC, Y.C, Y.DC, A.C, A.DC and A.P groups. (A) The UPGMA clustering tree Based on the Weighted UniFrac distance in phylum level. (B) The composition and relative abundance of bacterial communities in Kingdom, Phylum, Class, Family, Genus and Species levels. C.C, Children-Control group; C.DC, Children-Dental caries group; Y.C, Youth-Control group; Y.DC, Youth-Dental caries group; A.C. Adult-Control group; A.DC, Adult-Dental caries group; A.P, Adult-Periodontitis group.

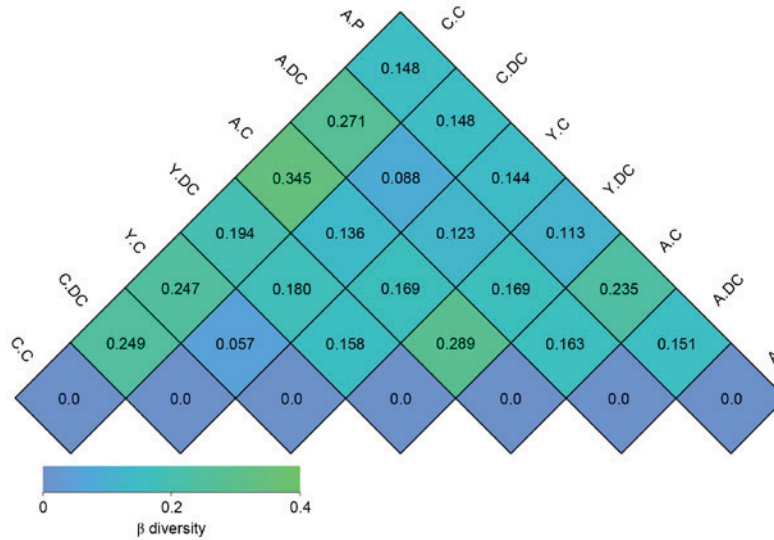


Figure 3. The heatmap of beta diversity index among C.C, C.DC, Y.C, Y.DC, A.C, A.DC and A.P groups. C.C, Children-Control group; C.DC, Children-Dental caries group; Y.C, Youth-Control group; Y.DC, Youth-Dental caries group; A.C. Adult-Control group; A.DC, Adult-Dental caries group; A.P, Adult-Periodontitis group.

from 32 to 19% in the youth group (Y.C vs. Y.DC). In the adult group, the dental caries made a slight increase in *Firmicutes* and a minor reduction in *Proteobacteria*, while the percentage of *Firmicutes* in the periodontitis group was greatly increased from 36 to 60%, and *Proteobacteria* was reduced from 50 to 25%.

As indicated in Fig. 3, similarities of 0.247 (C.C vs. Y.C), 0.345 (C.C vs. A.C) and 0.136 (Y.C vs. A.C) were obtained in the healthy group, and 0.180 (C.DC vs. Y.DC), 0.088 (C.DC vs. A. DC) and 0.169 (Y.DC vs. A.DC) were obtained in the dental caries group.

Compositions and relative abundance of bacterial communities in genus level. To further investigate the relative abundance in groups C.C, C.DC, Y.C, Y.DC, A.C, A.DC and A.P, the top 10 genera were clustered. As demonstrated in Fig. 4, the dominant bacterial genera in all groups were *Streptococcus* and *Neisseria*. When dental caries occurred,

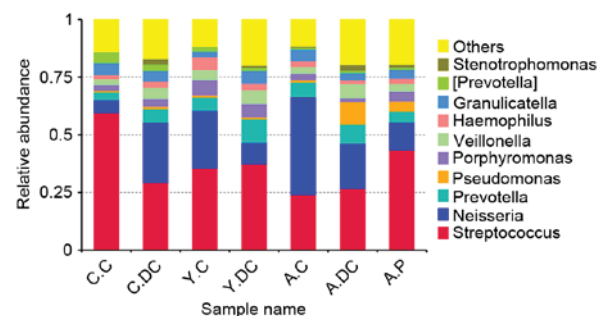


Figure 4. The relative abundance of the bacteria among C.C, C.DC, Y.C, Y.DC, A.C, A.DC and A.P groups at the genus level. C.C, Children-Control group; C.DC, Children-Dental caries group; Y.C, Youth-Control group; Y.DC, Youth-Dental caries group; A.C. Adult-Control group; A.DC, Adult-Dental caries group; A.P, Adult-Periodontitis group.

the relative abundance of *Streptococcus* reduced from 0.60 to 0.30 and *Neisseria* increased from 0.06 to 0.26 in the children

group. Notably, the dental caries posed a slight effect on the relative abundance of *Streptococcus*, while *Neisseria* received a dramatic reduction in the youth group (from 0.26 to 0.09) and the adult group (from 0.42 to 0.19).

Discussion

Oral diseases appear to be the outcome of multiple microorganisms, and the complexity of the microbial community in the oral cavity has hampered the identification of a single etiological agent for dental caries (25). Although it has been demonstrated that *Streptococcus sobrinus* and *Streptococcus mutans* are acidogenic and serve an important role in caries initiation, the molecular techniques revealed that a high proportion of samples from cavities do not contain *Mutans streptococci*, whereas other acid-producing bacteria, e.g. *Lactobacillus*, *Actinomyces* or *Bifidobacterium*, existed (26,27). Therefore, it is important to investigate the actual microbial diversity in oral disease as a cooperation of microorganisms, rather than as individual organisms or species in the human mouth (28).

In previous studies, high-throughput sequencing had been used to investigate the microbiota in human and canine oral cavities (5,19), which may ignore the importance of the age bracket on the tooth development and oral diseases. Moreover, the authors suggested that the low sample size in their studies may overlook inter-animal variations, as well as temporal changes that possibly occur, and that larger studies were required (5). To avoid the biodiversity loss during the DNA extraction process (the small size of each sample will make a huge loss of DNA) and to low the temporal changes of the oral microbiota, all the samples in the same age bracket were mixed and the mean number of 188,856.4 usable sequences (6-9 folds greater than the single sample in previous studies) and the saturated Chao1 index and Shannon index (Table I) ensured their reliability for the future analysis.

In Fig. 1, the Venn figure demonstrated that a mean of 315 OTUs were obtained in each group, and 73, 64, 53, 19 and 18 common OTUs belonging to *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria* and *Fusobacteria*, respectively, were identified in healthy people. As is already known, *Firmicutes* can survive in extreme conditions and made up the largest portion of the gut flora involved in energy resorption and obesity (29), *Bacteroides* are related to the metabolism of fat by absorbing nutrition and producing short-chain fatty acids (22), and *Proteobacteria* area major group of gram-negative bacteria (which includes a wide variety of pathogens) responsible for nitrogen fixation (30). Most of bacteria in these three phyla are regarded as pathogenic microorganisms, therefore the traditional treatment strategy to eradicate all the microorganisms in oral cavities using antibiotic drugs may not be suitable, as the 'pathogens' composed primarily of oral microorganisms and may serve a key role in oral health.

Based on the weighted UniFrac distance in phylum level, *Firmicutes*, *Proteobacteria* and *Bacteroidetes* accounted for >95% of the total sequencing number in each group, and their total occupancy received little change between healthy and disease conditions (Fig. 2). However, the reduction of *Firmicutes* from 75 to 45% and the increase of *Proteobacteria* from 10 to 39% indicated the dental caries in the children group characterized by the raising of *Firmicutes*. Conversely,

the dental caries made an increase of *Firmicutes* and a reduction of *Proteobacteria* in youth and adult groups, indicating that the age bracket was crucial for tooth development and microbial development. Considering the immature tooth development in the children group (6-18 years old) and the physiological depression in the adult group (35-60 years old), the microbial community in the youth group may be the most reasonable microbiota. Moreover, the high dissimilarity of 0.345 (C.C vs. A.C) in healthy patients and the high similarity of 0.088 (C.DC vs. A.DC) in diseased patients also confirmed the instability of the children and adult groups, when compared with the youth group (Fig. 3).

To further investigate the relative abundance among groups, the top 10 genera were clustered in Fig. 4, and *Streptococcus* and *Neisseria* were identified as the dominant bacteria genus in all groups. As with Fig. 2, the children group was characterized with the increase of *Neisseria*, while the youth group (from 0.26 to 0.09) and the adult group (from 0.42 to 0.19) were characterized by the reduction in *Neisseria*. The well-distributed bacteria were beneficial for the host and defended against invading pathogens (31), thus the more even microbial distribution in the Y.C group also confirmed their beneficial potential for pathogenic invasion.

In the present study, the microbial community of the human oral cavity had been explored using high-throughput sequencing, and these results indicated that the oral microbial communities were mainly composed of traditional 'pathogenic bacteria', and their ratio had been largely influenced by human age and presence of oral diseases. Therefore, it is necessary to further investigate the actual microbial diversity in healthy people and oral disease patients on a larger scale and identify the bacteria at species level; the unique bacteria should be screened and separated to study their role in maintaining the eco-balance of the oral cavity.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (grant nos. 91639106, 81270202 and 91339113 to H-B Xin, 31560264 and 81503364 to Tingtao Chen and 21461015 to Xiaolei Wang); the National Basic Research Program of China (grant no. 2013CB531103 to Hongbo Xin); the Open Foundation Of Hubei Key Laboratory Of Lipid Chemistry And Nutrition (grant no. 201602 to Tingtao Chen); the Science Foundation of Jiangxi Provincial Department of Education (grant nos. KJLD14010 and 20153BCB23035 to Xiaolei Wang and 20151BBG70239 to Yan Shi); and, the major program of Natural Science Foundation of Jiangxi Province (grant no. 20161ACB21002 to Xiaolei Wang).

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