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Decrease of oral microbial diversity might correlate with radiation esophagitis in patients with esophageal cancer undergoing chemoradiation: A pilot study

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Abstract

Objective: Radiation esophagitis (RE) is a common treatment-emergent adverse event that leads to significant morbidity and mortality in patients with unresectable esophageal cancer undergoing chemoradiotherapy. In the present study, we aimed to investigate the correlation between oral bacterial diversity and RE in patients with esophageal cancer undergoing chemoradiotherapy.

Methods: This study included 21 patients undergoing chemoradiotherapy, and 10 patients undergoing chemotherapy for pathologically confirmed squamous cell esophageal cancer between July 2018 and February 2019 at Shandong Cancer Hospital. Oral mucosal swabs were collected from 10 patients without RE, 11 patients with grade 1 RE, and 10 patients with grade ≥2 RE. The RE grade was based on the Common Terminology Criteria for Adverse Events (v5.0). Oral bacterial diversity was assessed by 16S rRNA gene sequencing. Permutational multivariate analysis of variance was carried out to determine whether the overall bacterial community composition differed among the groups. Linear discriminant analysis effect size was used to analyze species with significant differences among groups. Metagenome content was predicted using phylogenetic investigation of communities by reconstruction of unobserved states.

Results: The overall bacterial community composition was similar among the three groups. The abundance of the phylum Bacteroidetes was decreased in patients with RE compared with those without RE (linear discriminant analysis value >4, $P < 0.05$). There were significant functional differences in the flora between the patients without RE and patients with grade 1 RE, as well as between the patients without RE and patients with grade ≥2 RE ($P < 0.05$).

Conclusion: A decrease in oral bacterial diversity might be correlated with RE in patients with esophageal cancer undergoing chemoradiotherapy. These findings warrant further studies for the verification and elucidation of the underlying mechanisms, which will provide a theoretical basis for the prevention and treatment of RE.

KEYWORDS
16S rRNA gene sequencing, oral microbiota, radiation esophagitis, squamous cell esophageal cancer

1 | INTRODUCTION

The incidence and severity of radiation esophagitis (RE), a common treatment-emergent adverse event in patients with squamous cell esophageal cancer undergoing chemoradiotherapy, are higher in those receiving radiotherapy in combination with chemotherapy, whereas limiting the radiation dose can affect long-term therapeutic outcomes.[1,2] Recent studies have also suggested that radiation modality,[3] dose–volume parameter,[4] fraction scheme,[5] and certain human genes[6] are significant clinical factors that influence RE.

The treatment of RE is primarily based on symptomatic treatment, including anti-inflammatory agents and analgesics, as well as active surveillance.[7] Associations between human microbiota and radiation-induced injury have been reported in the gut,[8-11] and some studies have explored the regulation of microbiota composition for the treatment of radiation complications.[12,13] Th esophagus, similar to other luminal organs of the digestive system, provides a favorable environment for bacterial colonization. Oral microbiota directly participates in the composition of esophageal microbiomes because of migration or blood transportation.[14-16] Importantly, oral microbiota has been implicated as a potential contributor to the pathogenesis of RE. Understanding the association between oral microbiota and RE might provide better strategies for the prevention and treatment of radiation complications. However, the relationship between oral microbiota and RE remains unclear.

Supported by recent advances in next-generation sequencing with 16S rRNA gene amplicon analysis, the relationship between oral microbiota and systemic diseases, including diabetes, inflammatory bowel disease, obesity, and cancer, has been widely recognized and characterized.[17] Therefore, we carried out a prospective study using high-throughput sequencing to explore the characteristics of oral microbiomes of patients with RE, and elucidate the association between RE and oral microbiomes.

2 | METHODS

2.1 | Patients and groups

The present prospective study included adult patients who were treated with chemoradiotherapy or chemotherapy for pathologically confirmed squamous cell esophageal cancer between July 2018 and February 2019 at Shandong Cancer Hospital, Jinan, Shandong, Chia. Oral mucosal swabs were collected from patients with RE after the cumulative radiotherapy dose reached 20–30 Gy; these patients were categorized into two groups, including those with grade 1 RE (RE.1 group) and grade ≥2 RE (RE.2 group), respectively, according to the Common Terminology Criteria for Adverse Events (v 5.0). The patients who received chemotherapy were categorized as the non-RE (NRE) group. All patients gave their written informed consent. All data were prospectively analyzed in an anonymized form. The study was approved by the committee of Shandong Cancer Hospital, Jinan, China.

2.2 | Inclusion and exclusion criteria

The study included adult patients treated at Shandong Cancer Hospital regardless of age, sex, and weight. Those with oral, esophageal, gastrointestinal, or immune disorders other than primary esophageal cancer, and those with a history of treatment with antibiotics, immune modulators, hormone drugs, and microbiota regulators within 1 month before the collection of oral mucosal swabs were excluded. Information on age, sex, diagnosis, and treatment plan were recorded for all patients.

2.3 | Collection of oral mucosal swabs

All patients were strictly screened according to the inclusion and exclusion criteria. The patients were not allowed to eat, smoke, or drink within 1 h before sampling to maintain no other affected factors. The patients were asked to rinse their mouths two or three times with clean water for to ensure a clean oral cavity free of foreign bodies. Two mucosal swabs were collected from each patient, and all samples were stored in tubes at −80°C.

2.4 | Microbiome assay

Total genomic DNA from samples was extracted using cetyltrimethylammonium bromide. The concentration and purity of DNA were analyzed on 1% agarose gels, and the DNA samples were diluted to 1 ng/μL using sterile water. Barcoded amplicons covering the V3 + V4 region of *16S rRNA* were generated using the primers 341F, 5′-CCTAYGGGRBGCASCAG-3′ and 806R,5′-GGACTACNNGGGTATCTAAT-3′. Polymerase chain reaction (PCR) was carried out in a 30-μL reaction volume with 15 μL of Phusion High-Fidelity PCR master mix (New England Biolabs, Ipswich, MA, USA), 0.2 μmol/L each of forward and reverse primers, and approximately 10 ng template DNA. Thermal cycling conditions were as follows: initial denaturation at 98°C for 1 min; 30 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, and elongation at 72°C for 30 s, followed by final extension at 72°C for 5 min. After PCR, equal volumes of 1 × loading buffer and PCR products were separated by

electrophoresis on 2% agarose gel for detection. PCR products were mixed in equal density ratios. Next, the mixed PCR products were purified using a GeneJET gel extraction kit (Thermo Scientific, Waltham, MA, USA). Sequencing libraries were generated using the Ion Plus Fragment Library kit (Thermo Scientific) following the manufacturer's recommendations. The library quality was assessed using a Qubit 2.0 fluorometer (Thermo Scientific). Finally, the library was sequenced on an Ion S5 TM XL platform, and 400–600-bp single-end reads were generated.

2.5 | Sequence data processing

Single-end reads were assigned to samples based on unique barcodes and truncated by cutting off the barcode and primer sequences. Quality filtering on raw reads was carried out under specific filtering conditions to obtain high-quality clean reads according to the Cutadapt (v 1.9.1; National Bioinformatics Infrastructure, Uppsala, Sweden) quality-controlled process.[18] The reads were compared with the SILVA reference database[19] using the UCHIME algorithm[20] to detect chimeric sequences, which were then removed[21] to obtain clean reads. All clean reads were clustered into operational taxonomic units (OTUs) with 97% identity threshold using the UPARSE pipeline (v 7.0.1001; posted by Robert Edgar, an independent investigator, Tiburon, California, USA).[22] Annotation of taxonomic information was carried out based on the Mothur algorithm using the SILVA reference database, with a threshold set at 0.8–1.[19] To examine the phylogenetic relationship of different OTUs and differences in dominant species among different samples (groups), multiple sequence alignments were carried out using the MUSCLE program, Tiburon, California, USA (v 3.8.31; posted by Robert Edgar, an independent investigator, Tiburon, California, USA).[23] Finally, the abundance of information on OTUs was normalized using a standard sequence number corresponding to the sample with the least number of sequences. All subsequent analyses of α and β diversities were carried out based on the normalized data.

2.6 | Statistical analysis

In the present study, alpha diversity; that is, within-subject diversity, was assessed by measuring the Chao1 index and the Shannon diversity index, calculated in 100 iterations of rarefied OTU tables of 5000 sequence reads per sample. This depth was chosen to sufficiently reflect sample diversity while retaining all participants. Differences in alpha diversity among the groups were analyzed using Wilcoxon's rank-sum test.

Beta diversity; that is, between-subject diversity, was assessed at the OTU level using unweighted[24] and weighted[25] UniFrac distances calculated by the QIIME platform.[26] Principal coordinate analysis[27] was carried out to obtain principal coordinates and visualize complex, multidimensional data. Permutational multivariate analysis of variance was used to determine differences in the overall bacterial community composition according to the case status.

TABLE 1 Characteristics of enrolled esophageal squamous cell carcinoma cases and groups

Characteristics	Squamous cell carcinoma			
	NRE ($n = 10$)	RE.1 ($n = 11$)	RE.2 ($n = 10$)	Total cases
Age (years)				
<60	4	4	5	13
≥60	6	7	5	18
Sex				
Male	8	8	8	24
Female	2	3	2	7
Tumor location				
Cervical	1	2	2	5
Upper thoracic	2	2	5	9
Middle thoracic	4	5	1	10
Lower thoracic	3	2	2	7
TNM staging				
I	0	1	0	1
II	1	3	1	5
III	8	7	9	25
IV	1	0	0	1
Radiotherapy (IMRT)				
Involved field	–	5	3	12
Selected field	–	6	3	9
Chemotherapy				
Taxanes + platins	8	8	5	21
5-Fu + platins	2	0	4	6
5-Fu	0	3	1	4

5-Fu, 5-fluorouracil; IMRT, intensity modulated radiation therapy; NRE, non-radiation esophagitis; RE.1, grade 1 radiation esophagitis; RE.2 grade ≥2 radiation esophagitis; TNM, tumor–node–metastasis.

Linear discriminant analysis effect size was used to analyze species showing significant differences among groups. Species with significant differences in abundance among groups were determined using the non-parametric Kruskal–Wallis rank-sum test. The contribution of each group of different species to the differences in effect was estimated by linear discriminant analysis. The default filter value for the linear discriminant analysis score in the present study was 4.

Metagenome content was predicted using PiCRUSt (Curtis Huttenhower team, Boston, Massachusetts, USA).[28] Because PiCRUSt gene content is precomputed for the Greengenes database of 16S rRNA genes, in the present study we carried out closed-reference OTU picking against the Greengenes database before PiCRUSt. Next, the sequenced microbial composition was mapped to the database to predict the metabolic function of the flora. Finally, we used Student's *t*-test to assess differences in gene function among the groups.

Data analysis and visualization were carried out using R (v 2.15.3; R Foundation for Statistical Computing, Vienna, Austria). A *P*-value of <0.05 was considered to show statistical significance.

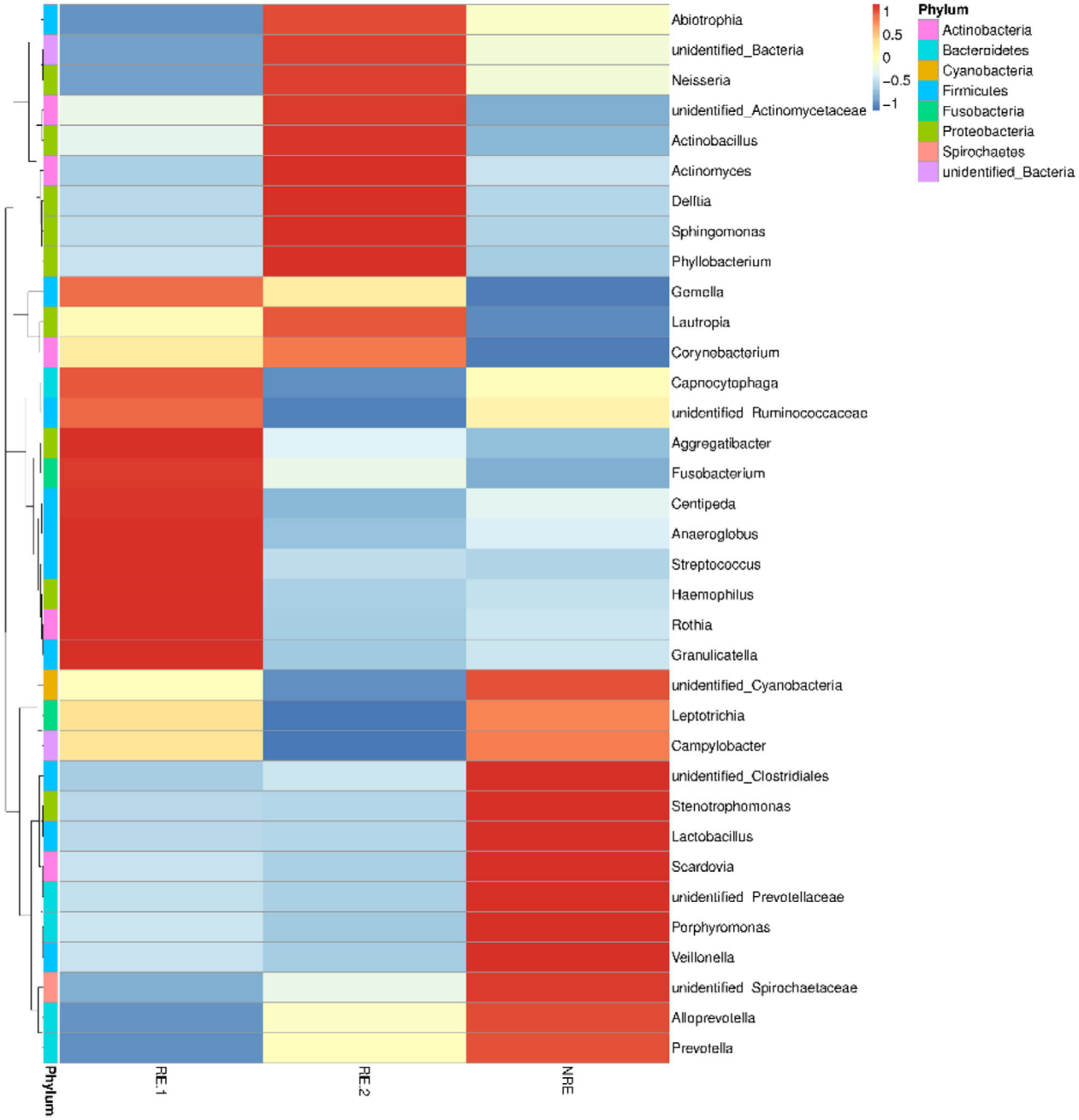

FIGURE 1 Cluster heatmap for species abundance between groups at the genus level

3 | RESULTS

The present study comprised of a total of 31 patients, including 10, 11, and 10 patients in the NRE, RE.1, and RE.2 groups, respectively. Clinicopathological characteristics, including age, sex, pathology, tumor location, TNM staging, and treatment strategy, were comparable among the groups (Table 1).

A total of 2770 OTUs obtained in the present study were annotated in the SILVA database. The proportion of phylum-level OTUs was 93.86%, whereas the proportion of species-level OTUs was 49.93%. Genera with abundances ranked among the top 35 were selected, and a heat map was constructed by clustering from two aspects of samples and species to visually determine which species gathered more or contained less in which samples or groups (Figure 1). The dominant genera included *Streptococcus*, *Neisseria*, and *Phyllobacterium*.

To evaluate the diversity and richness of bacteria in the samples, the Chao1 and Shannon indices were calculated. The mean values of the Chao1 and Shannon indices were 450.3 and 4.8 for the NRE group, 575.8 and 4.8 for the RE.1 group, and 639.4 and 4.7 for the NRE group, respectively. The OTU richness measured by the Chao1 index did not significantly differ for the following comparisons: NRE versus RE.1, NRE versus RE.2, and RE.1 versus RE.2 ($P = 0.42$, 0.38, and 0.25, respectively). Furthermore, the OTU diversity measured by the Shannon index did not significantly differ for the following comparisons:

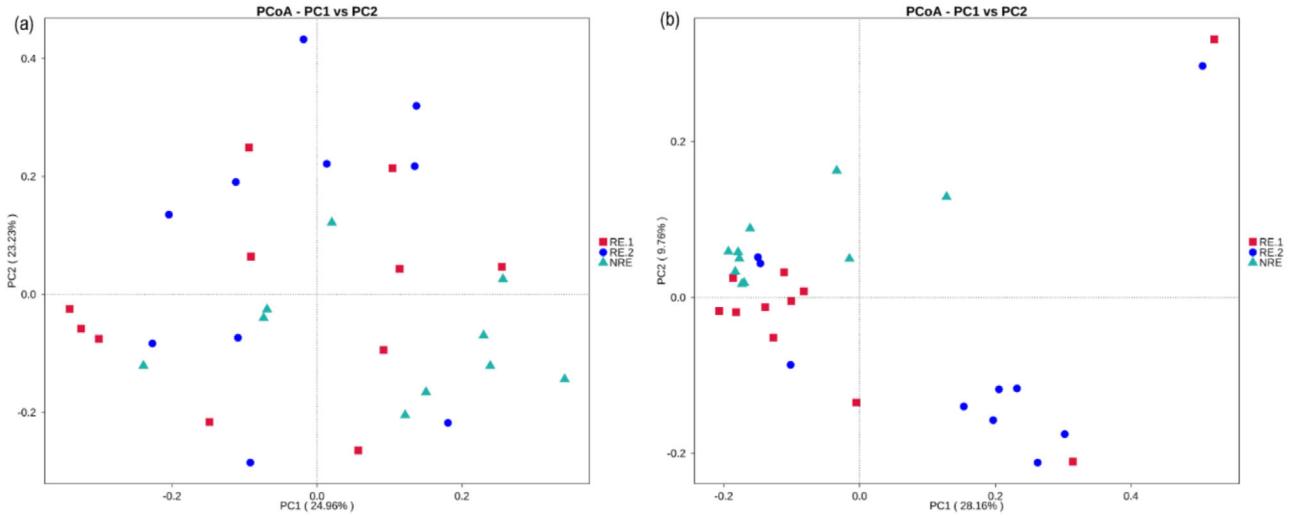

FIGURE 2 Distribution of samples shown by the two most significant coordinates for (a) weighted Unifrac and (b) unweighted Unifrac distances

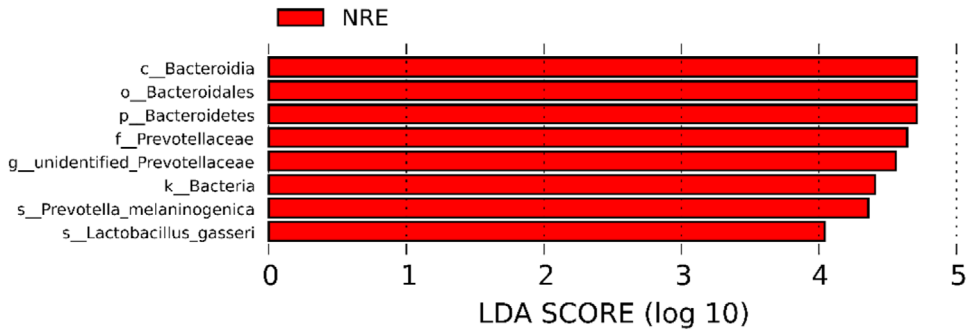

FIGURE 3 Linear discriminant analysis (LDA) value distribution histogram. NRE, non-radiation esophagitis

NRE versus RE.1, NRE versus RE.2, and RE.1 versus RE.2 ($P = 0.70, 0.45$, and 0.70, respectively).

We compared the overall bacterial community composition using weighted and unweighted UniFrac distance matrices, and applied principal coordinate analysis to ordinate the matrix (Figure 2). Based on the weighted UniFrac distance, the samples in each group showed a scattered distribution. Based on the unweighted UniFrac distance, an obvious aggregation was observed among samples within the same group in addition to an obvious separation between the groups. Permutational multivariate analysis of variance for differences in the community structure among groups showed no significant differences for the following comparisons: NRE versus RE.1, NRE versus RE.2, and RE.1 versus RE.2 ($P = 0.08, 0.15$, and 0.71, respectively). However, the linear discriminant analysis effect size for RE.1, RE.2, and NRE (Figures 3 and 4) showed the presence of several species with significant differences in abundance among the groups, including *Prevotella melaninogenica*, Bacteroidales, Bacteroidetes, *Lactobacillus gasseri*, Prevotellaceae, and Bacteroidia, all of which belonged to the phylum Bacteroidetes. This finding suggested that the abundance of these species decreased significantly in the RE.1 and RE.2.

Based on the OTU tree in the Greengenes database and the genetic information on the OTUs, we obtained gene function prediction spectrum of the whole bacteria. There were significant differences in the function of genes related to membrane transport, cellular processes and signaling, and enzyme families between the RE.1 and NRE groups (Figure 5). Furthermore, there were significant differences in genes related to lipid metabolism, enzyme families, and metabolic diseases between the RE.2 and NRE groups (Figure 6).

4 | DISCUSSION

In the present prospective study examining the relationship between oral microbiota and RE, we did not observe significant associations between RE and overall bacterial diversity or composition. However, we found that the abundance of Bacteroidetes was decreased in the RE.1 and RE.2 groups compared with that in the NRE group. Furthermore, compared with that in the NRE group, there were significant functional differences in the RE.1 and RE.2 groups. These findings suggest a nominal correlation of several bacterial species with RE.

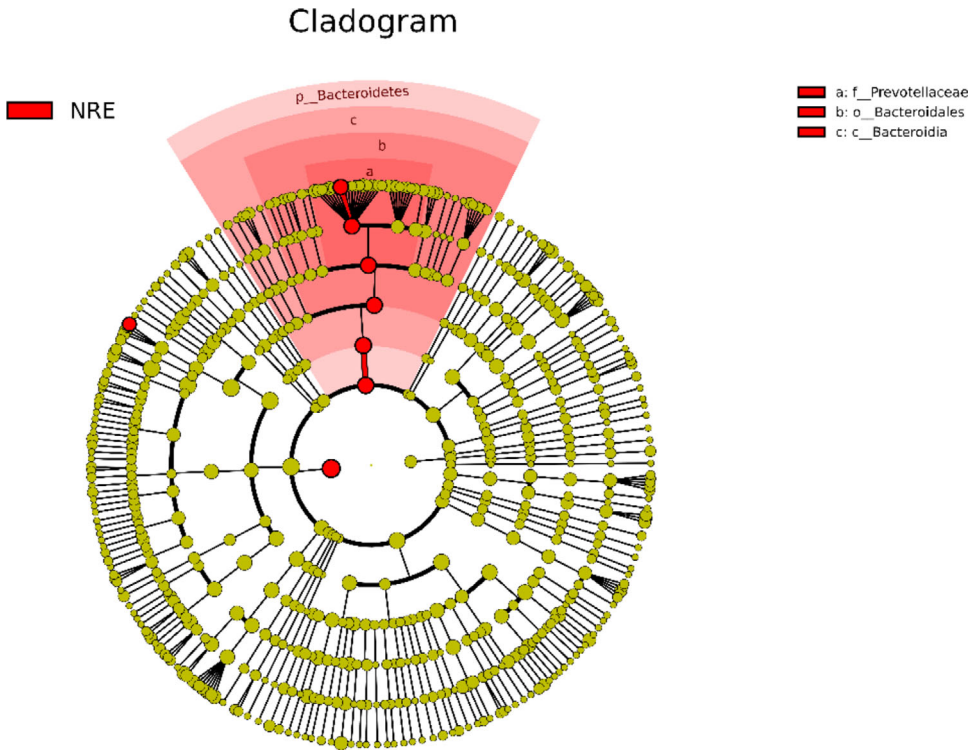

FIGURE 4 Phylogenetic distribution for biomarkers. NRE, non-radiation esophagitis

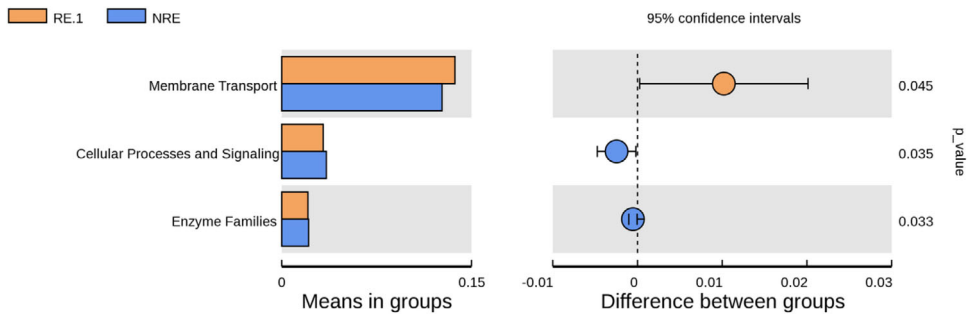

FIGURE 5 The *t*-test for functional difference between grade 1 radiation esophagitis (RE.1) and non-radiation esophagitis (NRE)

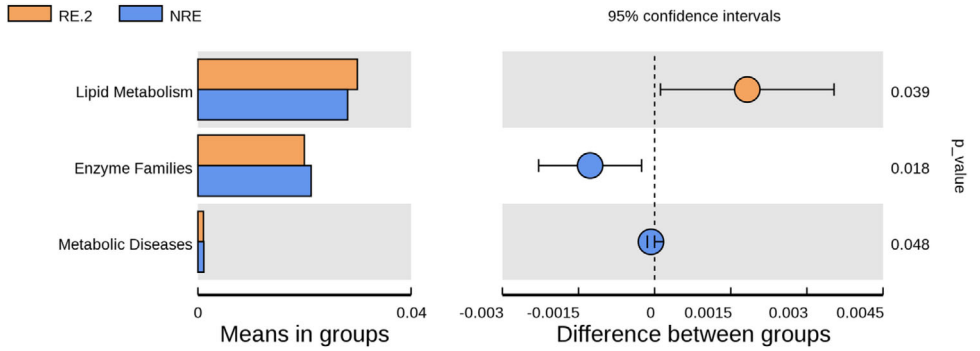

FIGURE 6 The *t*-test for functional difference between grade ≥2 radiation esophagitis (RE.2) and non-radiation esophagitis (NRE)

The microbiota profile varies among different oral habitats; however, the oral mucosal microbiota of most individuals is similar and tends to be fairly stable with age. These factors might have contributed to the minimal differences observed in the overall microbiota diversity among the groups. The attenuated abundance of oral Bacteroidetes in patients with RE might have contributed to the significant functional differences among the groups. As one of the dominant phyla in the digestive tract of healthy adults, Bacteroidetes play a key role in the host immunity system.[29] A recent study showed that *Bacteroides* participate in the development of inflammatory bowel disease by expressing an adenosine triphosphate-binding cassette exporter and lipoprotein, thereby activating the nuclear factor- κB signaling pathway.[30] In contrast, *Bacteroides* colonization of germ-free mice can correct the underdevelopment of the immune system.[31] In addition, *Bacteroidetes* are involved in metabolic syndrome and the regulation of the gut–brain axis, with interesting therapeutic implications in mood impairment and neurological disorders.[29] Our predictive analysis of metagenomic function based on the Kyoto Encyclopedia of Genes and Genomes database showed significant functional differences in membrane transport, cellular processes and signaling, and enzyme families between the NRE and RE.1 groups, and in lipid metabolism, enzyme families, and metabolic diseases between the NRE and RE.2 groups. In contrast, there were no significant differences between the RE.1 and RE.2 groups. These functional differences might be associated with the decrease in the abundance of Bacteroidetes. However, further studies are warranted to determine if Bacteroidetes plays a potential mechanistic role in inflammation caused by radiation, particularly whether its absence is associated with RE.

Host genetics plays a key role in determining the composition of oral microbiota.[32] Although the composition of the microbiota is unique to the individual, it is not constant, and there is >20% interpersonal variability in microbiota, which is influenced by factors such as diet, drugs, and anthropometric factors. To date, studies have shown that microbiota affects physiological, as well as pathological, processes in the context of digestion, nutrient absorption, and regulation of endocrine, neurological, and immune systems through different mechanisms.[33] These studies raise the possibility that the composition of human microbiota can be modulated through changes in diet, drugs, and environment. Early studies have constantly attempted to pretreat radiation-induced intestinal injury by changing the composition of intestinal microbiota[12,13] through mechanisms, such as the activation of Toll-like receptors (TLRs). TLRs can specifically recognize and bind conserved pathogen-related molecular patterns to trigger intracellular signal pathways, subsequently leading to cell proliferation, cell cycle regulation, and production of cytokines, thereby playing an important role in innate immunity. The potential role of TLRs in radiation-induced injury was explored in mice using pretreatment with a TLR ligand, which attenuated radiation injury.[34,35] Combined with the results of the present study, we boldly propose that modified probiotics containing Bacteroidetes might be applicable in a wide range of clinical settings for the prevention and treatment of radiation injury, which certainly should be verified by further animal experiments.

The strengths of the present study include the prospective design, comprehensive 16S rRNA gene sequencing, and inclusion of three groups However, the present study had several limitations. Lack of oral hygiene of the participants hampered the determination of whether individual oral condition was an independent risk factor that ultimately might have affected the statistical results. Inconsistency between the endoscopic and clinical grade of RE might have contributed to the lack of significant differences between the patients with different grades of RE, and endoscopic diagnosis might be a more reliable or accurate approach.[2,7] Furthermore, the collection of microbiota samples was limited to a specific time point or a limited time range in the present study, whereas microbiota is a dynamic process during radiotherapy. Therefore, examining long-term dynamic changes in the oral microbiota of each patient might be a more appropriate approach to elucidate its role in RE. In addition, the number of patients was small, limiting the statistical power to detect significant associations, and the study participants were enrolled in one study institution.

A decrease in oral bacterial diversity might be correlated with RE in patients with esophageal cancer undergoing chemoradiotherapy.

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CONFLICT OF INTEREST

The authors declare that they have read the article and there are no competing interests.

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