Tissue source may affect the esophageal flora in patients with esophageal squamous cell carcinoma

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Abstract. The aim of the present study was to provide a theoretical basis for the selection of standard sampling methods in the study of the esophageal microbiota in patients with esophageal squamous cell carcinoma (ESCC) by comparing the differences in bacterial communities between surgical and endoscopic esophageal mucosal tissues. A total of 72 patients with ESCC who were diagnosed at Taihe Hospital (Shiyan, China) between July 2018 and July 2019 were selected to participate in the present study. The sequence V_4 hypervariable region was amplified, and Illumina HiSeq sequencing was performed to analyze the differences between the two groups. The Shannon and Chao1 indices of the postoperative esophageal cancer tissue group samples (Group A) were higher than those of the esophageal mucosa tissue samples (Group B), and the difference was statistically significant ($P<0.05$). The Simpson index of Group A was higher than that of Group B, but the difference was not significant ($P>0.05$). The β diversity analysis demonstrated that the overall composition of the flora of the two groups was not significantly different. Linear discriminant analysis effect size analysis showed that the abundance of *Megasphaera*, *Actinobacteria*, *Enterobacteriaceae* and *Enterobacteriales* in Group A was significantly higher than that in Group B, but the abundance of *Mogibacteriaceae* in Group B was significantly higher than that in Group A. The top 60 species were selected using the random forest method to establish a model. The error rate of the prediction model constructed using the random forest method was 22.59%. The receiver operating characteristic (ROC) curve analysis confirmed that the present model was reliable and could effectively distinguish between the two groups of samples (area under the curve, 0.86). The source of the sample should be considered in studies investigating the esophageal flora. Considering the increased richness and improved uniformity of postoperative tissue microbiota compared with the mucosal group, it was predicted that postoperative tissue may be more conducive to the study of esophageal cancer microbiota.

Introduction

Among malignant tumors, esophageal carcinoma (EC) ranks seventh in terms of the global incidence and sixth in terms of mortality (1). This type of cancer includes two main pathological types: Esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (2). China accounts for approximately half of all ESCC cases worldwide (3). In China, esophageal cancer ranks sixth in terms of incidence rate among malignant tumors and fourth in terms of the number of deaths (4), and ESCC accounts for >90% of all EC cases (5).

As part of the tumor microenvironment, microorganisms may participate in tumor development by inducing chronic or persistent inflammation (6). The human microbiota includes trillions of bacteria, archaea, fungi and viruses that interact with the human body (2), and are distributed in the skin, respiratory tract, oral cavity and gastrointestinal tract (3), with >70% of the human microbiota located in the gastrointestinal tract (7). However, the microecological composition of each part is not uniform, and different parts of the gastrointestinal tract may have specific microecological communities (8).

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Abbreviations: ESCC, esophageal squamous cell carcinoma; EC, esophageal carcinoma; OTUs, operational taxonomic units; NMDS, nonmetric multidimensional scaling

Key words: ESCC, postoperative tissue, esophageal mucosa, 16S ribosomal RNA, high-throughput sequencing

Sex, obesity, age, food, host genetic background, environment, antibacterial drugs and other factors affect microbial structures (9-13). Furthermore, different methods of material extraction may affect research results on the digestive tract flora (14). Given the close relationship between gut microbiota and human health, studying gut microbiota is helpful for the diagnosis, assessment and prognosis evaluation of diseases (15). The microflora in the digestive tract is related to the occurrence and development of ESCC (16). The changes in the esophageal flora should be studied or specific bacterial changes should be detected, and these studies may be beneficial for the early diagnosis, evaluation and favorable prognosis of ESCC (17-19). The sampling methods for research on the flora that causes esophageal diseases include saliva collection, oropharyngeal swabs, esophageal mucosal swabs, endoscopic biopsies, endoscopic mucosal resection specimens, surgical biopsies after esophageal surgeries, esophageal string tests and Cytosponge devices (18,20-23).

The microbial composition may vary depending on the sampling method and tissue source, and the microbial community composition of the different segments of the digestive tract may exhibit variations (24). Therefore, the selection of samples for microbial analysis is crucial for research, and the sampling method may affect the results of gastrointestinal microbiota research. Studies on the esophageal flora of patients with ESCC remain in their infancy and, to the best of our knowledge, the most suitable type of samples for this disease is unknown (25-27).

The advantages and disadvantages of different sampling methods, and their effects on exploring the relationship between esophageal microbiota and different esophageal diseases still require further research. The aim of the present study was to provide a theoretical basis for the selection of standard sampling methods in the study of esophageal microbiota in patients with ESCC by comparing differences in the bacterial flora between surgical and endoscopic esophageal mucosa tissues.

Materials and methods

Sample source. A total of 72 patients with ESCC who were diagnosed via digestive endoscopy and thoracic surgery at Taihe Hospital (Shiyan, China) between July 2018 and July 2019 were selected to participate in the present study. The patients were divided into the postoperative tissue group (Group A) and the esophageal mucosa group (Group B) based on the different sample sources of esophageal cancer tissue. Group A comprised 27 esophageal cancer postoperative tissue samples, and Group B comprised 45 esophageal mucosa samples. Patients in group A ranged in age from 36 to 77 years (median, 62.5 years), while patients in group B ranged in age from 37 to 85 years (median, 65.4 years) (Table I).

For patients with ESCC, the following inclusion criteria were applied: Age ≥18 years; pathological diagnosis of ESCC; without metabolic diseases (such as diabetes), hyperlipidemia or other infectious diseases; good general condition; no intake of antibiotics, acid suppressants or probiotics within the past 2 months; balanced diet and no special dietary habits; and no serious liver, kidney and immunodeficiency diseases. The exclusion criteria were as follows: Use of drugs affecting the microecology of the esophagus in the past 2 months; complications of metabolic or infectious diseases; presence of tumors other than ESCC; incomplete data; and not considered suitable for inclusion by the researchers (such as individuals with severe picky eating, long-term alcohol abuse and recent oral disease).

The study protocol was reviewed and approved by the Taihe Hospital Ethics Committee (approval no. 2018KS020; Shiyan, China), and written informed consent was obtained from all patients before they were allowed to participate in the present study. Furthermore, the present study was conducted in accordance with the provisions of The Declaration of Helsinki.

Sample collection. Esophageal mucosal tissue samples were obtained during endoscopic examination. Gastroscopy was performed 6-8 h after fasting and warm water was used for gargling before examination. After the esophageal tumor lesions were found, four to eight specimens were collected with sterile biopsy forceps for examination. Two specimens were marked, placed in sterile cryopreservation tubes and frozen in -196˚C liquid nitrogen for temporary storage, and then transferred to a -80˚C refrigerator for long-term storage. The remaining tissues were fixed in 10% neutral buffered formalin at room temperature for 24-48 h, and sent to the pathology. Fixed tissue samples were dehydrated using a series of graded alcohol solutions (70, 95 and 100% ethanol) to remove moisture from the tissue. Alcohol was removed from dehydrated tissues with xylene to make the tissue transparent, and then the tissue was embedded and placed in paraffin. The treatment of surgical specimens was the same as for endoscopic mucosal tissue, and appropriate samples were chosen for follow-up studies in accordance with the inclusion criteria. The selected samples were quickly transferred to a -196˚C liquid nitrogen tank for temporary storage, and then transferred to a -80˚C refrigerator for long-term storage.

DNA extraction. The DNA of the sample was extracted with an UltraClean® Microbial DNA Isolation Kit (15,800; Mo Bio Laboratories, Inc.) using the sodium dodecyl sulfate lysate freeze-thaw method. The purity and quantity of the DNA were determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc.). The sample was frozen at -20˚C for later use.

16S ribosomal DNA sequencing. The V_4 region of the 16S ribosomal RNA gene was amplified by PCR. The primers included 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). The PCR system (50 μl) comprised the following: 25 μl Phusion High-Fidelity PCR Master Mix (M0531; New England BioLabs, Inc.), 3 μl each of forward/reverse primers (10 μM), 10 μl DNA template and 9 μl double-distilled water. The thermocycling conditions were as follows: Pre-denaturation at 98˚C for 30 sec, followed by 25 cycles of denaturation at 98˚C for 15 sec, annealing at 58˚C for 15 sec and extension at 72˚C for 15 sec, and a final extension at 72˚C for 1 min. The amplification products of each sample were detected by electrophoresis on a 1% agarose gel at 100 V for 40 min. The UVI gel imaging system (Thermo Fisher Scientific, Inc.) was used for image capture and recording, and DNA electrophoresis did not show mixed bands

Table I. Basic information of included patients with esophageal squamous cell carcinoma.

		Sex			Age, years			
Group	No.	Male, n	Female, n	P-value[a]	Minimum	Maximum	Mean	P-value[b]
Group A	27	23	4	0.607	36	77	62.5	0.251
Group B	45	38	7		37	85	65.4	

[a]Fisher's exact test; [b]unpaired Student's t-test. Group A, esophageal cancer postoperative tissues; Group B, esophageal mucosa group.

and tails, indicating that the purity of DNA fragments was good and there was no obvious degradation. The gel recovery kit (DP219-03; Tiangen Biotech Co., Ltd.) was used to recover and purify the DNA of the target strip. The Qubit® dsDNA HS Assay kit (Q32854; Invitrogen; Thermo Fisher Scientific, Inc.) was used to accurately quantify the recovered DNA, and parallel sequencing was performed following mixing of the samples (the same amount of library was taken from each sample). The library amplification products were analyzed for fragment length using an Agilent 2,100 Bioanalyzer (Agilent Technologies, Inc.) and High Sensitivity DNA Kit (5,067-4,626; Agilent Technologies, Inc.), and a Qubit 3.0 Fluorometer (Invitrogen; Thermo Fisher Scientific, Inc.) was used to measure the library concentration. The final concentration of the library on the machine was 1.8 pM. Paired-end 150-bp mode sequencing was performed on the library using an Illumina HiSeq 4,000 platform (Illumina, Inc.) and a HiSeg 3,000/4,000 SBS Kit (300 cycles; FC-410-1003; Illumina, Inc.). Sequencing was completed at Shanghai Biotecan Pharmaceuticals Co., Ltd.

Operational taxonomic units (OTUs) clustering and species annotation. OTUs were analyzed with V search version 2.4.4 (28) and clustered with a similarity of 97%. Representative sequences were annotated on the basis of the SILVA128 database (29). The abundance and classification of the OTUs were recorded.

Bioinformatics analysis and statistical analysis. Quantitative insights into microbial ecology (version 1.8.0; http://qiime.org/) and R (www.r-project.org; version 3.2.0) were used to analyze the data. α diversity indices, including Chao1, Shannon, Simpson and abundance-based coverage estimator, were calculated. The abundance and uniformity of OTUs were compared, and the UniFrac distance was calculated (30). Principal coordinates analysis and nonmetric multidimensional scaling (NMDS) plots were generated for the β analysis of the sample flora structure. The Vegan package (version 2.5-3; https://github.com/vegandevs/vegan/releases) in R (v3.2.0) software, MEGAN 4 (31,32) and Graphical Phylogenetic Analysis (version 1.1.3) were used to visualize the groups and abundances (33). Venn diagrams were generated using the Venn Diagram module of the R software (v3.2.0) to visualize common and unique OTUs between groups.

The Wilcoxon rank-sum test in the R 3.2.0 software package was used to compare the differences in microbial communities at various taxonomic levels between two groups. Species bearing significant differences between groups were selected using linear discriminant analysis (LDA) effect size (LEfSe) analysis (34) and an LDA value ≥2 was considered statistically significant with $P<0.05$. Random forest analysis was performed using the default settings of the random forest module in R 3.2.0 to compare the differences between groups, and the p ROC package was used for receiver operating characteristic (ROC) curve analysis (35,36).

The BugBase tool can be used for the prediction of the microbial phenotype, using OTU tables as input files to standardize the OTU tables. Subsequently, pre-processed databases and BugBase tools were used to automatically select thresholds to predict microbial phenotypes, and the abundance of each phenotype in each group was calculated and compared (37). The BugBase database was employed to predict the phenotypes of esophageal bacteria (38).

For intergroup comparison involving phenotypic content prediction, the Wilcoxon rank-sum test was used to compare the abundance information among group samples, and the P-value was obtained.

Statistical analysis was performed using SPSS 21.0 (IBM Corp.). Normally distributed continuous variables are presented as the mean ± standard deviation, nonnormally distributed continuous data are presented as the median (lower quartile, upper quartile) and microbial abundance was conveyed as a percentage. Fisher's exact test, unpaired Student's t-test and nonparametric Wilcoxon rank-sum tests were conducted for comparison. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Sample sequencing data. After clustering was performed with 97% similarity, 3,656 OTUs, including 2,926 in the esophageal cancer postoperative tissue group (Group A) and 2,772 in the esophageal mucosa group (Group B), and 2,042 in both groups, were obtained. A total of 884 OTUs were unique to Group A and 730 OTUs were unique to Group B (Fig. S1).

α diversity analysis. The Shannon and Chao indices of the postoperative tissue samples (Group A) were significantly higher than those of the esophageal mucosa tissue samples (Group B) ($P<0.05$). The Simpson index of Group A was higher than that of Group B, but the difference was not significant ($P>0.05$). These findings indicated that the diversity of the microbial flora in postoperative tissues was higher than that in the esophageal mucosa group (Fig. 1A).

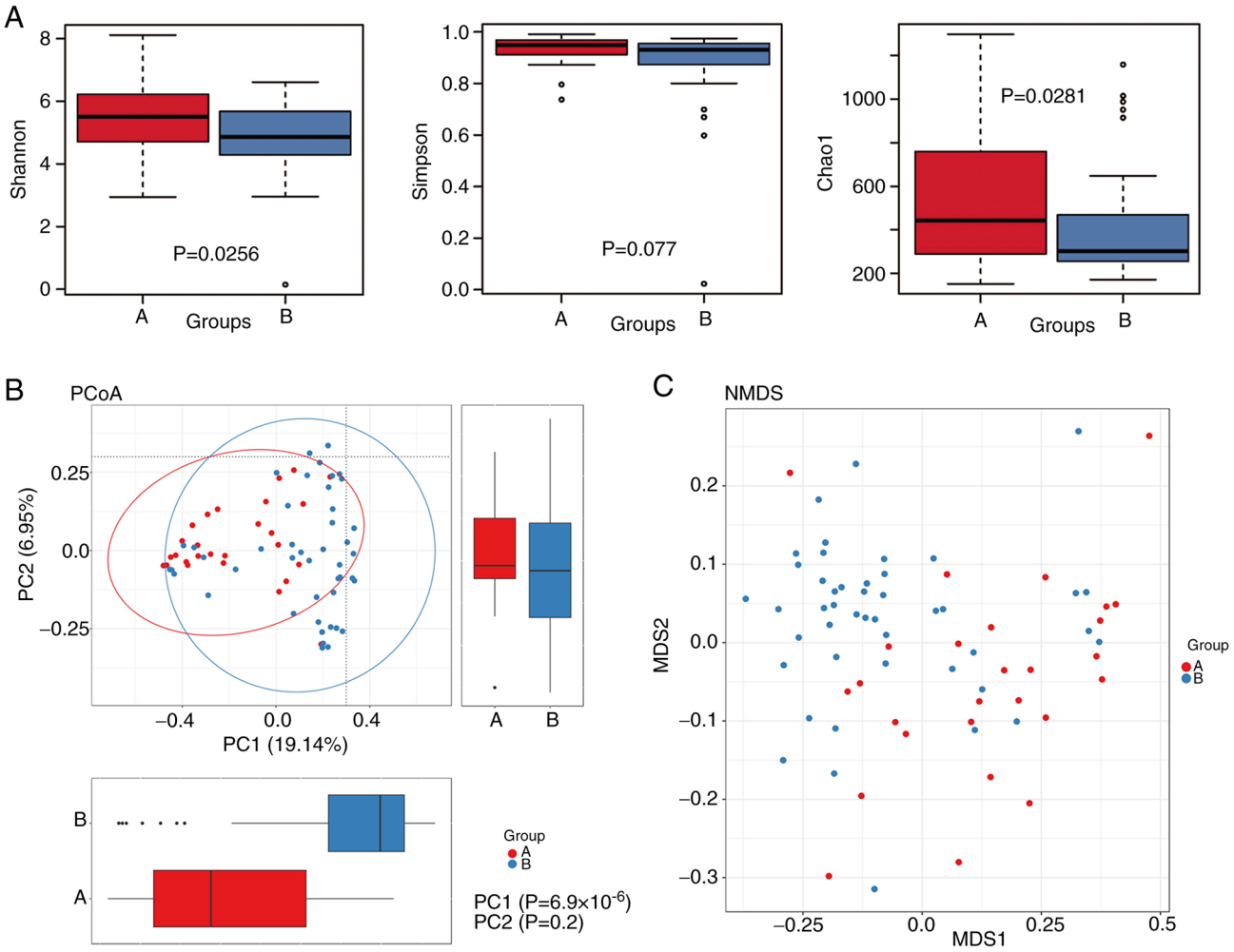

Figure 1. Comparison of α and β diversity of the esophageal flora after esophageal surgery (group A) and in the esophageal mucosa group (group B). (A) (A-1) Shannon, (A-2) Simpson and (A-3) Chao1 indices. The P-value is indicated at the top of each image. The abscissa indicates the name of the group, and the ordinate shows the α diversity index of the different groups. The box chart shows five statistics (minimum value, first quartile, median, third quartile and maximum value) as five lines from the bottom to the top. Outliers are indicated as 'o'. The P-value was calculated using the Wilcoxon rank sum test. (B) PCoA plots of the unweighted UniFrac distances of the variation in microbiota composition detected in the postoperative tissue group (Group A) and the esophageal mucosa group (Group B). The values of the two vectors are marked in the lower right corner. Each point in the figure represents a sample, red represents Group A, blue represents Group B, and the distance reflects the similarity of the samples. (C) Scatter plot of two groups of NMDS analysis results. Each point represents a sample, and points of the same color are from the same group. The distance reflects the similarity of the samples. In the plot, blue represents the esophageal mucosa group, and red corresponds to the esophageal cancer postoperative tissue group. NMDS, nonmetric multidimensional scaling; PC, principal component; PCoA, principal coordinate analysis.

β diversity analysis. Principal component (PC)1 and PC2 represented the potential factors influencing the deviation of the microbial composition of the two groups. For the two groups, PC1=19.14%, suggesting that the bacterial composition in the two groups was not significantly different (Fig. 1B). NMDS analysis showed that the overall flora of the two groups could not be clearly distinguished. This result demonstrated that the overall composition of the flora of the two groups was not markedly different (Fig. 1C).

Differential LEfSe analysis. The abundance of *Megasphaera*, *Actinobacteria (class level)*, *Actinobacteria (phylum level)*, *Enterobacteriaceae* and *Enterobacteriales* in the esophageal postoperative tissue samples (Group A) was higher than that in the esophageal mucosal tissue samples (Group B), but the abundance of *Mogibacteriaceae* in the esophageal mucosa tissue samples (Group B) was higher than that in the postoperative samples (Group A). The difference in microbial abundance between the two different tissues was statistically significant (P<0.05; Fig. 2A and B).

Characteristics of the esophageal flora of the two groups. There were differences in microbial composition between the two groups at the phylum and genus levels, as well as differences in classes, orders and families (Tables SI-SIII).

Analysis of the microbial flora composition at the phylum level. The two groups of samples were considerably different at the phylum level, and the five phyla with the most significant differences were identified. *Actinobacteria* and *Verrucomicrobiae* were more abundant in the postoperative tissue group than in the esophageal mucosa group. The abundance of *Fusobacteria*, *SR1* and *Spirochaetes* was significantly

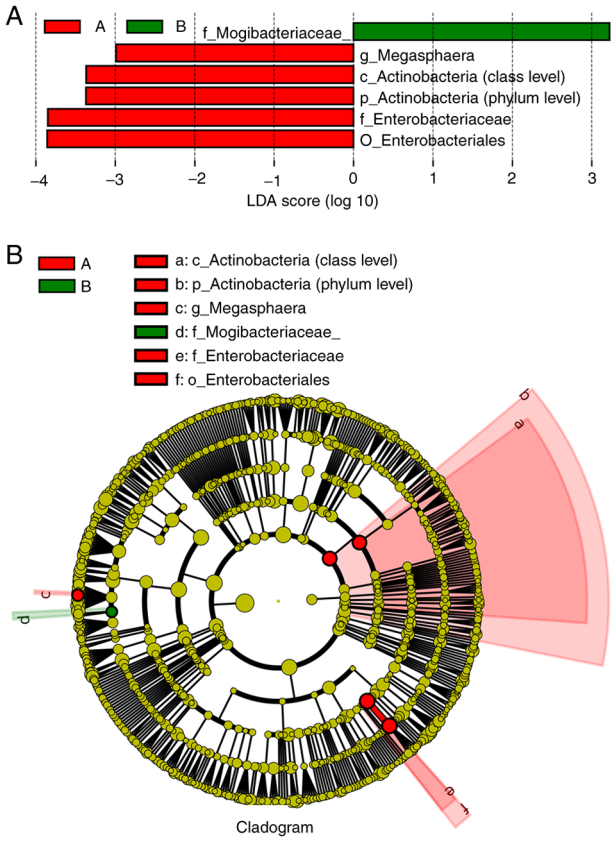

Figure 2. Species composition analysis of the esophageal flora after esophageal surgery (group A) and esophageal mucosa (group B). (A) Histogram of the distribution of LDA values of the two groups. (B) Cladogram of the representative microbial structure of the two groups. The dominant microbial classes of each group are represented by different colors. The diameter of each dot is proportional to the operational taxonomic unit abundance. Red and green represent the postoperative tissue group and esophageal mucosa group, respectively. Species with an impact value of >2 were identified as biomarkers using LDA. The cladogram depicts the abundance of species at the level of each taxonomic unit (circles from inside to outside and corresponding circle sizes) and their importance within a certain group. Only species with significant differences are shown in the figure. LDA, linear discriminant analysis.

lower in the postoperative tissue group than in the esophageal mucosa group (P<0.05; Table II).

Microbial flora composition analysis at the genus level. At the genus level, *Bifidobacterium*, *Collinsella, Bacteroides*, *Parabacteroides*, *Butyricimonas*, *Paraprevotella*, *Gemella*, *Enterococcus*,*Blautia*,*Coprococcus*,*Lachnospira*,*Roseburia*, *Faecalibacterium*, *Oscillospira*, *Ruminococcus*, *Megamonas*, *Megasphaera*, *Ruminococcus*, *Phascolarctobacterium*, *Sutterella* and *Akkermansia* were more abundant in the postoperative tissue group than in the esophageal mucosa group, whereas the abundance of *Porphyromonas*, *Prevotella*, *[Prevotella]*, *Catonella*, *Oribacterium*, *Peptostreptococcus*, *Selenomonas*, *Parvimonas*, *Fusobacterium*, *Leptotrichia*, *Ralstonia*, *Campylobacter*, *Actinobacillus* and *Treponema* in the former was significantly lower than that in the latter (P<0.05; Table III).

Predictive performance of the esophageal microbiome in two groups of patients (genus level). The random forest method is a machine learning method that can effectively classify and predict grouped samples. The bacterial genera that serve a major role in the classification performance in the classifier were arranged in descending order of their effects (Fig. 3A). The top 60 species were selected for the random forest method to establish a model. The error rate refers to the error rate of using the characteristics of the microbial community for random forest method prediction classification. The higher the error rate, the lower the accuracy of classification based on bacterial genus features, which may result in unclear bacterial genus features between groups. The error rate was 22.59% (Fig. 3A). The ROC curve confirmed that the forecasting model constructed by the random forest method was reliable and could effectively distinguish between the two groups of samples (area under the curve, 0.86; Fig. 3B).

Comparison of phenotype classification based on BugBase. The phenotype prediction using BugBase showed that the relative abundance of Gram-positive bacteria was higher in the postoperative tissue group than in the mucosal tissue group. By contrast, the relative abundance of Gram-negative bacteria in the postoperative tissue group was significantly lower than that in the mucosal tissue group (Fig. S2; P<0.05, Table IV). The two groups were similar under the following conditions: Aerobic, anaerobic, presence of mobile elements, facultatively anaerobic, forms biofilms, potentially pathogenic and stress-tolerant conditions, and the differences were not significant (P>0.05; Table IV).

Discussion

The normal human microbiota serves a role in human nutrition, drug metabolism, maintenance of the integrity of the intestinal mucosal barrier, immunomodulation and protection against pathogens (39). Changes in microbial community composition are related to numerous diseases, including tumors (40,41). Bacteria were first found in tumors over a century ago (42). Different tumor types have a unique flora; however, the characterization of tumor microbiomes is often challenging because of their low biomass (43). The microbiota, as a part of the tumor microenvironment, serves an important role in tumorigenesis and metastasis (44). However, the composition of microbial communities in different parts of the human body is not consistent

The amount of bacteria in the digestive tract is 10 times the total amount of human cells (45). Most bacteria have a specific spatial distribution and are not cultivable (46,47). The microbial communities in the mouth, esophagus and rectum vary in type and quantity (48). The composition of microbial communities may vary between different organs of the same individual and different parts of the same organ (41,48-51). Therefore, in microbial community research, the influence of organs and tissues on microbial communities needs to be considered. At present, the gut microbiota is the most extensively explored component of the digestive tract microbiota (52,53). Different sampling methods may affect the results of research examining microbial communities. In order to identify more reasonable sampling methods, scholars have conducted extensive research (54-58).

The esophagus contains numerous types of bacteria, and abundant florae can be found between the oropharynx and the stomach. Some esophageal florae in the stomach are

Table II. Significant differences in phylum levels between the two groups.

	Median (%)		IQR				
			P 25 (%)		P75 (%)		
Name	Group A	Group B	Group A	Group B	Group A	Group B	P-value[a]
Actinobacteria	2.330	1.319	1.318	0.504	3.727	3.074	0.030
Fusobacteria	0.683	4.017	0.246	1.358	1.345	7.050	<0.001
SR1	0.000	0.021	0.000	0.000	0.004	0.131	0.003
Spirochaetes	0.007	0.189	0.000	0.022	0.161	1.464	0.007
Verrucomicrobia	0.276	0.000	0.001	0.000	0.591	0.097	0.005

[a]Wilcoxon rank-sum test. IQR, interquartile range; Group A, esophageal cancer postoperative tissues; Group B, esophageal mucosa group.

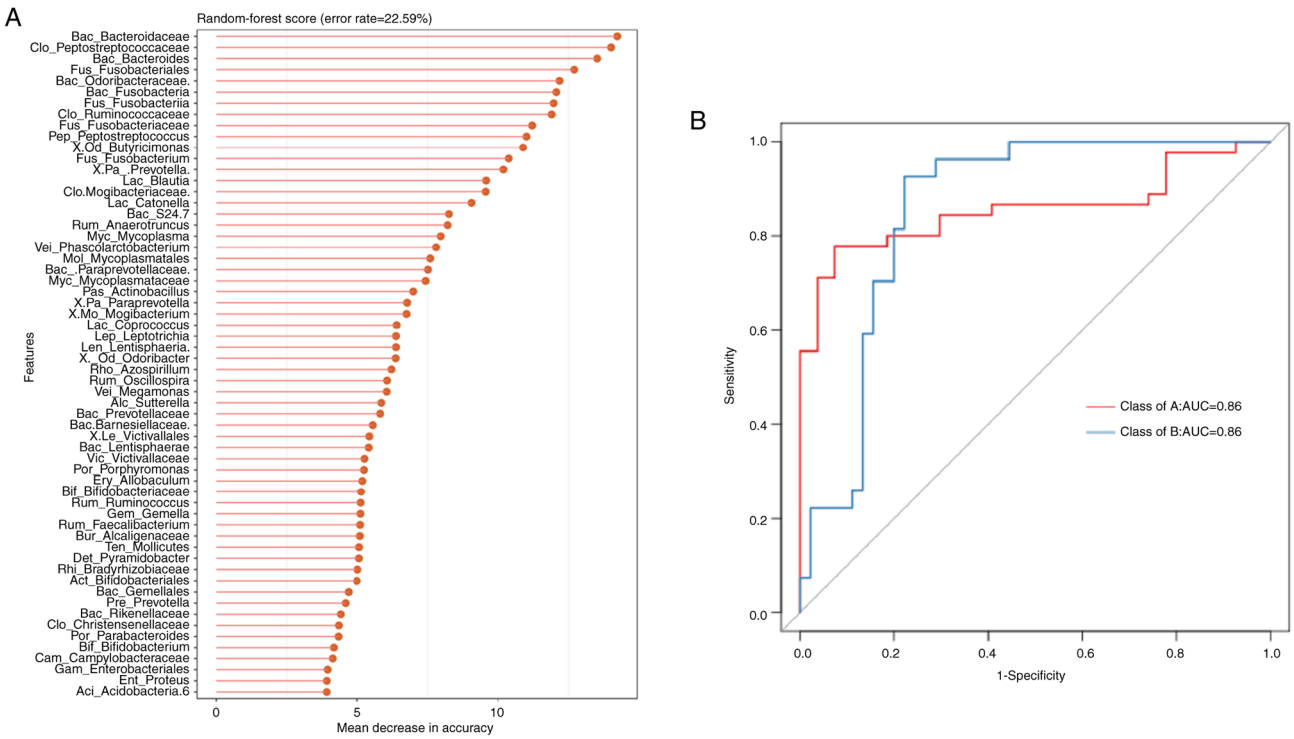

Figure 3. Comparison of the flora between groups and selection of species markers. (A) Species importance map. The abscissa reflects the importance level, and the ordinate indicates the species name sorted according to importance. The figure reflects the bacterial strains that serve a major role in the classification performance of the classifier, arranged in descending order of their effects. The figure shows the species at all levels. The higher the error rate value, the lower the accuracy of classification based on bacterial features, which may indicate that the bacterial features between the two groups are similar. (B) ROC curve analyzing the clinical accuracy of using differential bacteria obtained from the postoperative tissue group and the esophageal mucosa group. The point closest to the upper left of the ROC graph is the critical value with the greatest sensitivity and specificity. AUC, area under the curve; Class of, at the class level; ROC, receiver operating characteristic.

similar to those in the oral cavity, and the three different parts of the esophagus have no specific bacteria (20,59). The abundance of archaea and phages in a normal esophagus is low, and a normal esophagus also contains *Streptococcus*, *Prevotella*, *Veillonella*, *Clostridium*, *Haemophilus*, *Neisseria*, *Porphyromonas* and other bacteria (17,60). Shao *et al* (61) found that the microbial environment of ESCC is composed of Firmicutes, Bacteroidetes and Proteobacteria. The abundance of *Fusobacterium* in tumors is increased (3.2 vs. 1.3 %), whereas the abundance of *Streptococcus* is decreased (12 vs. 30.2%) compared with that in nontumor tissues (61).

Studies have been performed to improve the sampling methods of esophageal flora. Liu *et al* (15) reported that swabs and biopsies of patients with ESCC had similar microbial profiles. However, Gall *et al* (20) suggested that the amount of DNA recovered from a mucosal chip brush was greater than that from mucosal samples in esophageal adenocarcinoma. Okereke *et al* (62) studied Barrett's esophagus and confirmed that swabs obtained from the oropharynx or an endoscope could not replace biopsies of esophageal mucosa. Further research also demonstrated that mucosal biopsy should be used for the analysis of the esophageal flora (21).

Table III. Significant differences in genus levels between the two groups.

	Median (%)		IQR				
			P 25 (%)		P75 (%)		
Name	Group A	Group B	Group A	Group B	Group A	Group B	P-value[a]
Bifidobacterium	0.849	0.063	0.437	0.003	1.496	0.620	0.001
Collinsella	0.039	0.000	0.001	0.000	0.187	0.011	0.001
Bacteroides	12.194	0.907	7.543	0.176	27.594	4.786	<0.001
Parabacteroides	0.697	0.021	0.145	0.001	1.673	0.362	<0.001
Porphyromonas	0.043	0.518	0.015	0.095	0.218	3.505	<0.001
Prevotella	5.757	12.227	2.510	4.770	8.104	22.868	0.001
Butyricimonas	0.053	0.001	0.012	0.000	0.229	0.011	<0.001
Paraprevotella	0.156	0.000	0.015	0.000	0.235	0.025	<0.001
[Prevotella]	0.231	2.475	0.063	0.599	0.490	5.784	<0.001
Gemella	0.002	0.000	0.000	0.000	0.012	0.002	0.011
Enterococcus	0.075	0.010	0.002	0.000	0.177	0.069	0.020
Blautia	0.218	0.001	0.011	0.000	0.531	0.024	<0.001
Catonella	0.000	0.159	0.000	0.009	0.001	0.319	<0.001
Coprococcus	0.112	0.001	0.016	0.000	0.257	0.028	<0.001
Lachnospira	0.398	0.032	0.080	0.001	1.214	0.382	0.007
Oribacterium	0.001	0.052	0.000	0.000	0.031	0.215	0.013
Roseburia	0.314	0.002	0.038	0.000	0.893	0.272	0.002
Ruminococcus	0.276	0.000	0.001	0.000	0.434	0.069	0.003
Peptostreptococcus	0.007	0.220	0.002	0.044	0.109	0.945	<0.001
Faecalibacterium	0.436	0.007	0.089	0.000	1.320	0.117	0.001
Oscillospira	0.223	0.002	0.009	0.000	0.779	0.051	<0.001
Ruminococcus	0.604	0.030	0.124	0.000	0.969	0.390	<0.001
Megamonas	0.305	0.003	0.104	0.000	1.095	0.234	<0.001
Megasphaera	0.071	0.005	0.005	0.000	0.199	0.062	0.013
Phascolarctobacterium	1.037	0.001	0.278	0.000	2.109	0.329	<0.001
Selenomonas	0.013	0.679	0.000	0.069	0.209	4.561	<0.001
Parvimonas	0.001	0.032	0.000	0.004	0.013	0.239	0.001
Fusobacterium	0.408	2.577	0.101	0.996	1.251	5.611	<0.001
Leptotrichia	0.009	0.220	0.003	0.021	0.097	0.887	<0.001
Sutterella	0.773	0.015	0.224	0.000	1.290	0.434	<0.001
Ralstonia	0.000	0.001	0.000	0.000	0.002	0.020	0.014
Campylobacter	0.034	0.228	0.009	0.066	0.381	1.088	0.007
Actinobacillus	0.001	0.087	0.000	0.008	0.072	0.694	0.003
Treponema	0.007	0.189	0.000	0.022	0.161	1.458	0.006
Akkermansia	0.190	0.000	0.000	0.000	0.591	0.074	0.005

[a]Wilcoxon rank-sum test. IQR, interquartile range; Group A, esophageal cancer postoperative tissues; Group B, esophageal mucosa group.

α diversity can reflect the diversity of a microbial community (63). The Chao1 index describes the richness of a community and reflects the number of microbial members, such as OTUs, in a community. The Shannon and Simpson indices reflect the uniformity of a community and the abundance of its members (63). The present study revealed that the Chao1 and Shannon indices of the postoperative tissue group were increased compared with those of the mucosal tissue group. Although the Simpson index of the postoperative tissue group was higher than that of the mucosal tissue group, the difference between the two groups was not statistically significant, suggesting that the postoperative tissue flora was richer than the mucosal tissue flora, and the uniformity was good, indicating that the distribution of bacteria in the postoperative tissue group was uniform. The β diversity of the microbiome refers to the differences between samples in colony structures, which can be investigated at two sample sites, ecological communities or populations (64). The two groups of bacteria had a P-value >0.05, indicating that the diversity of the two groups was not significantly different.

Table IV. Comparison of phenotype classification based on BugBase.

Phenotype of prokaryotic microorganisms	Relative abundance with trait (median)		IQR				
			Relative abundance with trait (P25)		Relative abundance with trait (P75)		
	Group A	Group B	Group A	Group B	Group A	Group B	P-value[a]
Aerobic	0.080	0.103	0.038	0.041	0.153	0.213	0.603
Anaerobic	0.551	0.579	0.342	0.465	0.768	0.763	0.418
Contains mobile elements	0.362	0.348	0.244	0.247	0.409	0.496	0.945
Facultatively anaerobic	0.175	0.172	0.132	0.089	0.278	0.263	0.314
Forms biofilms	0.265	0.236	0.192	0.150	0.376	0.447	0.890
Gram-negative	0.589	0.667	0.482	0.513	0.687	0.785	0.039
Gram-positive	0.411	0.333	0.313	0.215	0.518	0.487	0.039
Potentially pathogenic	0.174	0.129	0.108	0.059	0.225	0.295	0.555
Stress tolerant	0.169	0.174	0.108	0.063	0.231	0.317	0.936

[a]Wilcoxon rank sum test. IQR, interquartile range; Group A, esophageal cancer postoperative tissue; Group B, esophageal mucosa group.

LEfSe analysis revealed that the flora of the two groups included different species. *Megasphaera*, *Actinobacteria*, *Enterobacteriaceae* and *Enterobacteriales* were more abundant in the postoperative esophagus tissues than in the mucosal tissues. *Mogibacteriaceae* was more abundant in the mucosal tissue group than in the postoperative tissue group. The bacterial species of the two groups were compared at the phylum and genus levels. The predominant phyla in the postoperative tissue group were *Actinobacteria* and *Verrucomicrobiae*. The dominant phyla in the mucosal tissue group were *Fusobacteria*, *SR1* and Spirochaetes.

Analysis at the genus level revealed different dominant bacteria in the two groups of flora. The different distributions of flora in the esophageal tissues can be explained as follows: The flora may participate in the occurrence and development of ESCC, and the abundance of bacteria changes with the tumor progression and invasion of ESCC (65,66). The differences between the two groups might be caused by variations in pH gastric acid, bile reflux, and other undetermined factors (61,67).

The random forest method was adopted in the present study, and the top 60 species were selected to establish a model. The reliability of the model was verified using ROC curve analysis, and the model could effectively distinguish between the two groups of samples. BugBase is a microecological component analysis tool that can identify high-level phenotypes present in microecological samples and make phenotype predictions. Phenotypic types include Gram-positive, Gram-negative, biofilm formation, pathogenicity, mobile elements, oxygen demand (including anaerobic bacteria, aerobic bacteria and facultative bacteria), and oxidative stress tolerance (39). The comparison of the BugBase phenotypes of the two groups showed differences in Gram-negative and Gram-positive bacteria, and this finding might be related to the aforementioned variation in the distribution of bacterial groups. In the human body, by understanding the microbial phenotype, more targeted treatments can be selected (68). The present study may provide a reference for the study of the microbiota of esophageal cancer.

Although flora activity is not the only factor in the pathogenesis of ESCC, dysbacteriosis may serve an important role in the occurrence and development of ESCC (69). The present study demonstrated that there were differences in the microbial composition between postoperative esophageal cancer tissues and esophageal mucosal tissues. The source of the sample should be considered in studies on the esophageal flora. Considering the increased richness and improved uniformity of postoperative tissue microbiota compared with the mucosal group, it was predicted that postoperative tissue may be more conducive to the study of esophageal cancer microbiota.

The present study had some limitations that can affect the interpretation of the results. First, the florae of different parts of the esophagus and postoperative tissues were not compared. Second, other sampling methods, such as endoscopic smear, were not applied. Third, as aforementioned, two types of sources of esophageal cancer tissue were included in the present study. However, the postoperative tissues and endoscopic biopsy tissues included in the study were not from the same patients. After the esophageal mucosal tissue was sampled, it was divided into two parts. One part was sent to the pathology department for further pathological examination, and the other part was frozen for further investigation. Only tissues confirmed by pathologists as esophageal cancer were included in the present study. Similarly, the patients included in the postoperative tissue group were all diagnosed with ESCC by pathologists. In the present study, the esophageal mucosal and postoperative esophageal cancer tissues were not obtained from the same individuals for two main reasons. First, some patients are diagnosed with esophageal cancer after they have completed gastroscopy and pathological examination, but they may no longer be suitable for direct surgery and instead choose radiotherapy, chemotherapy or immunotherapy. For these patients, only endoscopic tissue can be obtained and postoperative tissue cannot be obtained. Second, some patients

diagnosed with esophageal cancer may receive further surgical treatment at a hospital near where they reside instead, so it may not be possible to obtain postoperative samples. Similarly, some patients who have been diagnosed with esophageal cancer in other hospitals choose to undergo surgery at Shiyan Taihe Hospital (Shiyan, China). As these patients did not undergo gastroscopy examination at Shiyan Taihe Hospital, endoscopic esophageal mucosal tissues from these patients could not be obtained. Finally, the sample size of the present study was small and the study included only two types of tissue. Future studies should use a larger sample size and more types of esophageal tissue to determine the best collection method for evaluating esophageal samples. The present study included an analysis of the composition of esophageal microbiota in postoperative tissues and mucosal tissues of ESCC, and found that there were differences in microbial composition between the two types of tissues. The optimal potential biomarkers for distinguishing between the two tissues were screened. This may provide a reference for sample selection in future studies on the esophageal microbiome of patients with ESCC.

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Availability of data and materials

The data generated in the present study may be found in the Sequence Read Archive database under accession number Bioproject PRJNA779607 or at the following URL: https://www.ncbi.nlm.nih.gov/bioproject/?term=779607.

Authors' contributions

XBL, ZYG, QT and SXH contributed to the conceptualization of the study, and reviewed and edited the manuscript. XBL, JCM and ZYG wrote the manuscript. ZYG and JCM performed statistical analyses. JRZ, WX and HW collected clinical data and samples. QT and SXH contributed to funding acquisition and editing. XBL and QT confirm the authenticity of all the raw data. All authors revised the manuscript, and read and approved the final manuscript.

Ethics approval and consent to participate

The study protocol was reviewed and approved by the Taihe Hospital Ethics Committee (approval no. 2018KS020; Shiyan, China), and all patients received information concerning their participation in the study and provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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