Characterization of the Esophageal Microbiota in Patients with Esophagitis and Esophageal Squamous Cell Carcinoma

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Research

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Abstract

Background: Microbial imbalances have been well elucidated in esophageal adenocarcinoma, but few studies addressing the microbiota in esophageal squamous cell carcinoma (ESCC) and esophagitis (ES). We aimed to explore the associations of esophageal microbiota with these patients.

Results: A total of 68 individuals were enrolled (control = 21, ES=15, ESCC = 32). Microbial diversity was significant different between ESCC patients and healthy controls by Chao1 index, Shannon index and PLS-DA. *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Actinobacteria* and *Fusobacteria* were the five dominant bacterial phyla among three groups. *Megamonas*, *Collinsella*, *Roseburia* and *Ruminococcus_2* showed a significantly continuous decreasing trend from Normal to ESCC at the genus level. When compared with control group, decreased *Fusobacteria* at phylum level and *Faecalibacterium*, *Bacteroides*, *Curvibacter* and *Blautia* at genus level were detected. ESCC samples also displayed a striking reduction of *Bacteroidetes*, *Faecalibacterium*, *Bacteroides* and *Blautia* in comparison with ES patients. LEfSe analysis indicated a greater abundance of *Streptococcus*, *Actinobacillus*, *Peptostreptococcus*, *Fusobacterium*, *Prevotella* in ESCC groups.

Conclusions: Our study suggests a potential association between esophageal microbiome dysbiosis and ESCC and provides insights on a potential screening marker for esophageal cancer.

Introduction

Esophageal cancer is a highly invasive and rapidly growing global concern, ranking as the fourth leading cause of cancer related death, which contributes to a substantial public health burden [1]. The main histological types of esophageal carcinoma are esophageal adenocarcinoma (EAC) and esophageal squamous cell carcinoma (ESCC), with the latter predominating in the Chinese population [2]. Despite the introduction of multiple methods including surgery, chemotherapy, radiotherapy and combination of radiotherapy and chemotherapy, the prognosis of ESCC remains poor, with the overall 5-year survival rate of 30.3% [3, 4]. Therefore, elucidation of the pathogenesis underlying ESCC and characterization of novel biomarkers are important and would offer new therapeutic alternatives.

Esophagitis (ES), smoking, drinking, and heredity are the known risk factors related to ESCC [5, 6]. Recently, microorganisms have been linked to a significant number of gastrointestinal disease and several cancers. At least 38 trillion microorganisms colonize human gastrointestinal tract and associate with the immunological homeostasis. Increasing evidence have demonstrated that imbalance of certain species was involved in tumor occurrence and progression through producing carcinogenic toxins, dampening the immunity and damaging DNA structure. Although, esophagus is an important part of the upper digestive system, few studies have investigated the relationship between human esophageal microbiota and disease. Previous studies found that there was a significant decrease in bacterial counts and alterations in microbial communities in the GERD and Barrett's esophagus groups [7, 8]. Alteration of microbial diversity including decreased *Veillonella* and *Streptococcus* and higher level of *Lactobacillus*,

Enterobacteriaceae and *Akkermansia* are associated with EAC [9]. However, diverse esophageal microbiome in human ESCC are less well characterized.

In the present study, we aimed to assess and compare the diversity and composition of the microbiota between ESCC, ES and normal tissues. This might further illustrate the role of microbiota in the pathogenesis of ESCC.

Materials And Methods

Study participants

We recruited 15 esophagitis patients (ES group) and 21 healthy volunteers (normal group) at Nanjing First Hospital from 2018 to 2019. A total of 32 ESCC patients (ESCS group) well enrolled from Jinhu people's Hospital. Sampling from oesophagectomy specimens was done with a sterile scalpel blade (cutting down to submucosa) within 1 h of surgical resection. All samples were flash frozen in liquid nitrogen and stored at −80°C. To minimize the potential influence on the microbiota, all patients enrolled should not receive antibiotics, H_2 receptor antagonists, proton pump inhibitors and probiotics 1 month before sample collection. Patients who had received radiotherapy, chemotherapy, or/and prior surgery were excluded. The study protocol was approved by the institutional review board of Nanjing Medical University, and all experiments were performed in accordance with approved guidelines and regulations.

DNA extraction

Total genome DNA from samples was extracted using the QIAamp DNA Mini Kit (QIAGEN, Valencia, CA, USA) combined with the bead-beating method. The DNA concentrations of each sample were adjusted to 50 ng/μl for subsequent 16S rDNA genes analysis. The bacterial DNA samples were stored at −80 °C for sequencing.

PCR amplification

16S rDNA genes of V4 region were amplified used universal primers (F: 5'-GTGCCAGCMGCCGCGGTAA-3', R: 5'-GGACTACHVGGGTWTCTAAT-3') with a 6-bp barcode. All PCR reactions (including denaturation, annealing and elongation) were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). After electrophoresis of PCR products, samples with bright main strip between 400−450 bp were chosen for next mixing and purification with Qiagen Gel Extraction Kit (Qiagen, Germany). At last, sequencing libraries were generated and sequenced on an Illumina MiSeq PE-300 platform (Illumina, San Diego, USA). Barcodes and sequencing primers were trimmed before assembly.

Sequencing processing and analysis

The raw data were filtered with QIIME(v 1.8.0), discarding the reads which were dereplicated or shorter than 150bp. Filtered reads were clustered into operational taxonomic units (OTUs) assuming 97% similarity. Compared with the SILVA database（version 128), the species classification information of

each OTU was obtained. All raw reads were stored in NCBI Sequence Read Archive (SRA) database, and the accession number is PRJNA759579.

Statistical analysis

For continuous variables, independent t-test, White's nonparametric t-test, and Mann-Whitney U test were applied. For categorical variables between groups, Pearson chi-square or Fisher's exact test was used, depending on assumption validity. QIIME software was used to evaluate the α diversity by calculating the Shannon index and Simpson index. To compare the differences of diversity among groups, β diversity was tested by partial least squares discrimination analysis (PLS-DA). Linear discriminant analysis (LDA) effect size (LEfSe) was performed to find key microbes associated with different groups with the LDA threshold of 3. We used Phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) analysis to predict Kyoto Encyclopedia of Genes and Genomes (KEGG) biochemical pathways. Statistical analysis was performed using the SPSS V19.0 (SPSS Inc., Chicago, IL) and STAMP V2.1.3. GraphPad Prism version 6.0 (San Diego, CA) was used for preparation of graphs. A p value <0.05 was considered statistically significant.

Results

Baseline characteristics of participants

A total of 21 healthy volunteers, 15 ES patients and 32 ESCC patients were enrolled in this study. Demographic characteristics of all included individuals were shown in Table 1. High age was observed in ESCC groups when compared with healthy controls. There was no significant difference in sex, alcohol intake, smoking, diabetic background and family history of cancer.

Table 1 Clinical characteristics of enrolled patients and healthy controls

Characteristics	Normal (n=21)	ES (n=15)	ESCC (n=32)	p value
Age	47.85±12.01	55.60±11.53	55.97±11.62	0.040*
BMI (kg/m2)	24.06±3.78	24.57±2.92	25.89±4.06	0.200
Sex (male)	13	9	20	0.986
Smoker (Yes)	11	10	18	0.684
Alcohol consumption (Yes)	7	6	14	0.750
Diabetes (Yes)	4	7	9	0.214
Family history of cancer (Yes)	3	2	3	0.796

Microbial diversity and richness between three groups

After sequencing and quality filtering, more than 3.2 million tags and a total of 2134 OTUs were obtained with the dominant length of tags locating among 400-440bp (Figure 1A). To test the sequencing depth, we created the rarefaction curves and showed a reasonable amount of sampling (Figure 1B).

The microbial α diversity and β diversity were applied to analyze the microbiota biodiversity and composition among groups. We used Chao1 index and Shannon index to describe the community richness and diversity. A higher richness of microbiota was observed in ESC and ES group than in normal group according to the Chao1 index (ESCC VS normal, p=0.0001, ES VS normal, p=0.0012, Figure 2A). Compared with normal group, the Shannon index of the ESCC group decreased significantly, indicating a lower microbial diversity (p=0.0417, Figure 2B). Whereas, the ES group owned significantly higher Shannon index, in comparison with ESCC group (p<0.0001) and normal group (p=0.0022). Moreover, the Venn diagram indicated that 493 of the total 2134 OTUs were shared among the three groups, with 72, 219 and 871 OUTs were unique for normal, ES and ESCC group respectively (Figure 2C). About β diversity, Partial least squares Discriminant Analysis (PLS-DA) at the OTU level revealed a statistically significant clustering (Figure 2D), suggesting different microbial community structures.

The Changes of Esophageal Microbiota Composition between the three groups

As shown in Figrue 3A-C, each group showed different bacterial composition at the phylum, family, class and genus levels. We explored taxa distribution at the phylum, family and genus level to reveal distinctive characteristics of each group. *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Actinobacteria* and *Fusobacteria* were the five dominant bacterial phyla in the three groups.

Normal esophageal was composed mainly by *Firmicutes* (62.5%), *Proteobacteria* (18.2%), *Bacteroidetes* (13.9%), *Actinobacteria* (2.6%) and *Fusobacteria* (1.3%), plus another ~1.5% of unidentified bacteria. At the genus level, *Streptococcus* (24.3%) was the main contributor to the microbiota profile, followed by *Faecalibacterium* and *Bacteroides* (6.1% and 4.3%, respectively); other subdominant genera were *Lactobacillus*, *Neisseria*, *Curvibacter* and *Blautia*, accounting for about 3% each (Figure 3A-C).

ES group showed a significant decrease of *Firmicutes* (p=0.0370) together with a statistically significant robust increase of *Fusobacteria* (p=0.0280) and *Bacteroidetes* (p=0.0060) with its corresponding genus *Bacteroides* (p=0.0240) as compared to normal groups (Figure 3A-C).

ESCC samples also displayed a striking reduction in its microbial composition, such as in *Fusobacteria* (p=0.0010) at phylum level and *Faecalibacterium* (p=0.0010), *Bacteroides* (p=0.0090), *Curvibacter* (p=0.0010) and *Blautia* (p=0.0040) in comparison with normal groups at genus level. We observed an increasing tendency of *Streptococcus* in ESCC groups. When compared with ES groups, decreased *Bacteroidetes* (p=0.0010), *Faecalibacterium* (p=0.0010), *Bacteroides* (p=0.0010) and *Blautia* (p=0.0040) with overexpressed *Streptococcus* (p=0.0070) in ESCC tissues were identified (Figure 3A-C). In

addition, *Megamonas*, *Collinsella*, *Roseburia* and *Ruminococcus_2* showed a significantly continuous decreasing trend from Normal to ESCC at the genus level (Figure 3D).

Characterized Microbial Taxa Associated With ESCC Patients

We used multi-level LEfSe analysis to explore potential important microbe biomarkers for the groups in all taxa, and the results of LEfSe among the three groups demonstrated that 138 bacterial species abundance had statistically significant differences. There were 41, 45, and 52 taxa that were abundant in normal volunteers, ES patients, and ESCC patients respectively. Given the large number different bacterial species, we focused on the taxa with LAD socres > 4.0. As shown in Figure 4, at the genus level, increased *Streptococcus* (LDA score=4.9115, p=0.0021), *Actinobacillus* (LDA score=4.5193, p<0.0001), *Peptostreptococcus* (LDA score=4.3049, p<0.0001), *Fusobacterium* (LDA score=4.2109, p=0.0004), *Prevotella* (LDA score=4.0768, p=0.0020) were detected as powerful markers in ESCC patients. Particularly, *Streptococcus anginosus* at the species level (LDA score=4.0115, p<0.0001) showed a greater abundance in ESCC groups. Besides, we observed a high level of *Roseburia* (LDA score=4.0412, p=0.0001), *Faecalibacterium* (LDA score=4.4607, p<0.0001) and *Curvibacter* (LDA score=4.0812, p<0.0001) at the genus level, and *Alphaproteobacteria* (LDA score=4.2618, p=0.0002) at the class level in the normal individuals. *Bacteroides* (LDA score=4.6561, p=0.0002) and *Blautia* (LDA score=4.0883, p<0.0001) at genus level were abundant in ES patients (Figure 4).

Functional analysis of esophageal microbiota across groups

Finally, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) was conducted to predict the metagenomes and identify the KEGG pathways involved in each group.

Patients with ESCC showed a significant upregulation of microbial genes involved in signaling molecules and interaction, excretory system, cellular community, cell growth and death, membrane transport, energy metabolism, metabolism of other amino acids, nucleotide metabolism, folding, sorting and degradation, translation, glycan biosynthesis and metabolism, replication and repair, metabolism of cofactors and vitamins, while lipid metabolism, xenobiotics biodegradation and metabolism, cell motility, amino acid metabolism, carbohydrate metabolism, transcription, signal transduction were consistently down-regulated compared to normal groups (Figure 5A).

Microbiota associated to ES was characterized by a higher potential for excretory system, digestive system, folding, sorting and degradation, energy metabolism, glycan biosynthesis and metabolism, metabolism of cofactors and vitamins, while showed robustly reduced xenobiotics biodegradation and metabolism, lipid metabolism, cell motility, membrane transport, signal transduction in comparison with control-associated microbiota (Figure 5B).

Moreover, when comparing ES and ESCC tissues, ESCC-associated microbiota showed a significantly increased signaling molecules and interaction, infectious disease, cell growth and death, membrane

transport, nucleotide metabolism, folding, sorting and degradation, metabolism of other amino acids, translation, metabolism of cofactors and vitamins, and replication and repair. Conversely, it displayed a consistently decreased lipid metabolism, amino acid metabolism, cell motility, carbohydrate metabolism, transcription, biosynthesis of other secondary metabolites, signal transduction pathways (Figure 5C).

Discussion

Increasing evidence have shown the crucial roles of bacterial infections in tumor development, including esophageal cancers[10]. In China, ESCC constitutes more than 90% of all esophageal cancers[11]. Although imbalanced microbiome has been well elucidated in EAC, microbial alterations of ESCC were still inconclusive. In the present study, we profiled the structure of esophageal microbiota in ES, ESCC patients and matched control through 16S rRNA gene sequencing and predicted the functional changes. We found a lower microbial diversity in ESCC than in healthy controls, which were supported by previous findings[12], whereas other studies indicated a decreasing tendency or a higher diversity without significant difference[13–15]. Although studies conducted by Geng et al. and Natalia et al.[12, 16]suggested decreased richness of microbiota in ESCC patients, our study observed a contrary trend. It could be partly explained by factors that could affect microbial structure such as geographic area or the organ studied. In accordance with previous research, the β diversity was statistically different between the ESCC and normal groups[12, 14].The microbial dysbiosis of ESCC tissues was characterized by decreased *Faecalibacterium*, *Bacteroides*, *Curvibacter* and *Blautia* and increased *Fusobacteria*.

Bacteroides is a predominant member of the gut microbiota with Bacteroides fragilis as the most prevalent form, which was an opportunistic pathogen related to abdominal, soft tissues and bloodstream infections[17]. Later studies revealed that a subtype of *Bacteroides* could produce a heat-labile toxin named *Bacteroides fragilis* toxin. Secreted toxin promoted IL-18 production and cleared E-cadherin which lead to profound inflammation and epithelial homeostasis[18]. Indeed, higher levels of toxigenic *Bacteroides fragilis* strains have been reported in secretory diarrhea and various types of cancer including colorectal cancer, prostate cancer[19, 20]. It seems to be inconsistency between our study and previous reports when Cheng et al. have demonstrated significantly enriched *Bacteroides* in ESC[13]. However, this discrepancy might be explained by samples isolated from different sites and diverse diets of the individuals recruited. Meanwhile, recent findings also revealed the protective effect of *Bacteroides fragilis* in the development of colitis related colorectal cancer. Thus, we proposed that the *Bacteroides* owned bidirectional role in the oncogenesis, and future functional and theoretical investigation of was urgent to confirm its role in ESCC.

Specifically, a higher abundance of *Streptococcus anginosus* in ESCC tissues was identified. *Streptococcus anginosus*, as an oral bacterium was frequently found in oral cavity, gastrointestinal tract and genitourinary tract[21]. It composed up for 82% of patient-unique strains collected from hospitalized patients and was involved in purulent infections, including endocarditis[22, 23]. In addition, the presence

of *Streptococcus anginosus* have been reported in head and neck squamous cell carcinomas, gastric cancer, dysplasia of the esophagus and esophageal cancer tissues, which indicated the association of *Streptococcus anginosus* in the carcinogenic process[24, 25]. Viable *Streptococcus anginosus* isolated from esophageal cancer tissues could adhere to cultured epithelial cells and induce the mRNA expression of two CXC-chemokine genes, IL-8 and GRO. These results were supported by a higher content of inflammatory cytokines in esophageal cancer tissues[26]. Streptolysin S encoded by the sag gene cluster was supposed to be responsible for the cytotoxicity of *Streptococcus anginosus*[27]. The involvement in sulphur metabolism might be the alternative strategy for *Streptococcus anginosus* in the carcinogenesis[28]. However, more clinical strains were urgent to better verify the pathogenic genes and cytotoxicity in future studies. Interestingly, increased abundance of *Fusobacterium*, another common elongated anaerobic gram-negative bacteria of the oral cavity, was also observed in ESCC tissures. Similar results have been confirmed in previous studies[12, 13]. However, Wei et al. found no significant difference of *Fusobacterium* between ESCC and healthy control[15]. *Fusobacterium* caused periodontal disease and was related to the development of human cancers. Increased *Fusobacterium nucleatum* in esophageal cancer was a biomarker of poor clinical outcome[29]. Experimental studies have shown that *Fusobacterium nucleatum* could promote the carcinogenesis by induction of chemokines and activation of β-catenin signaling pathway[29, 30]. In our study, we failed to find the different abundance of *Porphyromonas gingivalis* among three groups, which contributed to the development of ESC through enhancing IL-6 secretion and promoting epithelial-mesenchymal transition[11]. Since esophageal microbiome was partly shaped by the oral microbiome that linked the periodontal disease to ESCC[31], it suggested the possibility of protection against periodontal disease to prevent oncogenesis. This hypothesis was partly supported by the findings that numbers of lost teeth and lifestyle factors, including alcohol use an d oral hygiene were related to increased risk of ESCC[32].

Apart from compositional changes in bacterial taxa, we also predicted alteration in functions across groups. Metabolic reprogramming is a hallmark of cancer. Dysregulated metabolites including glucose, lipids and amino acids have been reported in upper gastrointestinal cancers. For example, increased lactic acid, citrate and glyceraldehyde were related to the gastric cancer and esophageal cancer, although opposite changes were documented by other studies. In our study, we observed a reduction of carbohydrate and amino acid metabolism, which suggested potential underlying mechanism of ESCC.

Nevertheless, there were several limitations about this study should be addressed. First, relative small sample size of each group limited the generalizability of our findings, and larger studies would provide more credible results. Additionally, current cross-sectional study urged a follow prospective trial to fully demonstrate the role of microbiota in esophageal diseases. Lastly, we just examined the difference in microbiota compositions among the three groups, where exploration of molecular mechanisms of the microbiota are essential.

Conclusions

Overall, our data investigated the microbiota spectrum of ESC patients and demonstrated significant difference in the microbial diversity and richness between ESC patients and normal subjects. We provided Streptococcus, Actinobacillus, Peptostreptococcus, Fusobacterium, Prevotella as potential markers in ESC patients. These observations might yield novel therapeutic targets of ESC. Further studies are required to confirm our results and elucidate mechanisms of the causal relationship.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Contribution statement

Zongdan Jiang, Zhenyu Zhang and Shukui Wang conceived, organized and supervised the project, and proofread the manuscript. Zongdan Jiang and Ziyang Shen collected and analysed the data, and drafted the manuscript. Jun Wang supervised statistical analysis. All authors approved the final version of the manuscript. All authors critically revised and approved the final version.

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Figures

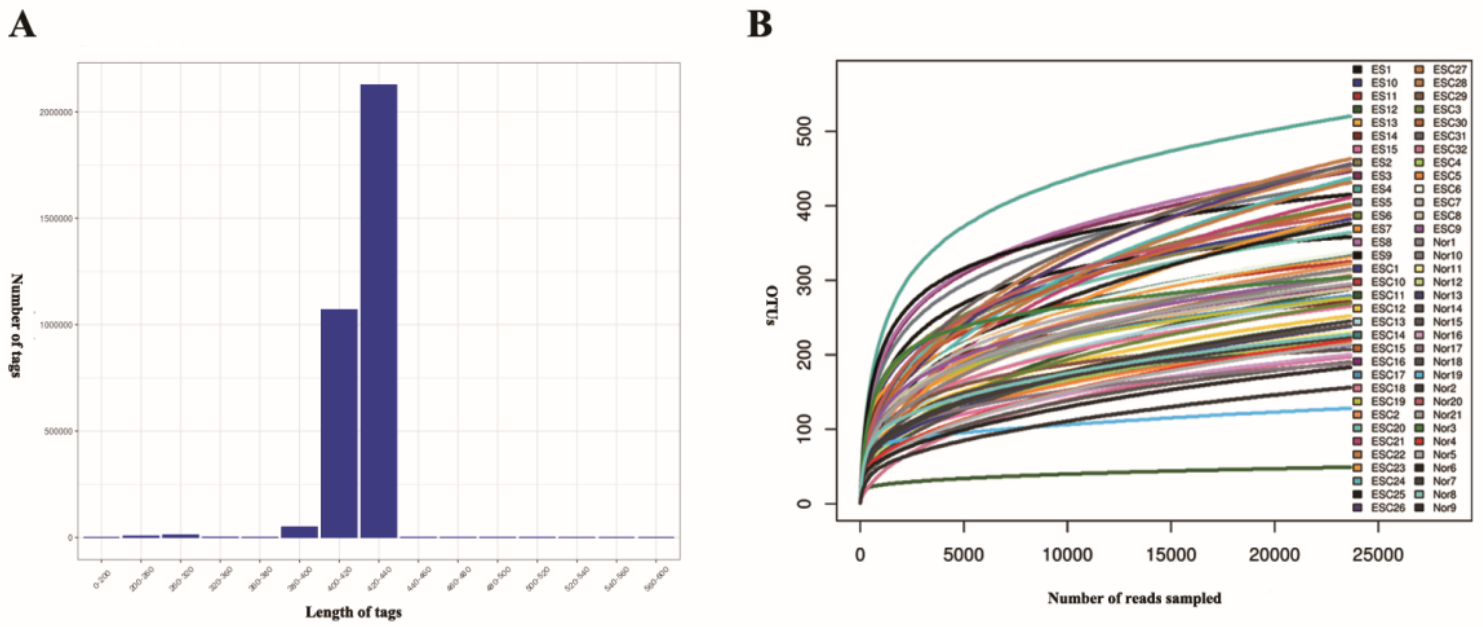

Figure 1

Quality Control and Basic Analysis (A) The abscissa is the sequence length of tags, and the ordinate is the number of tags. (B) Rarefaction curves for OTUs.

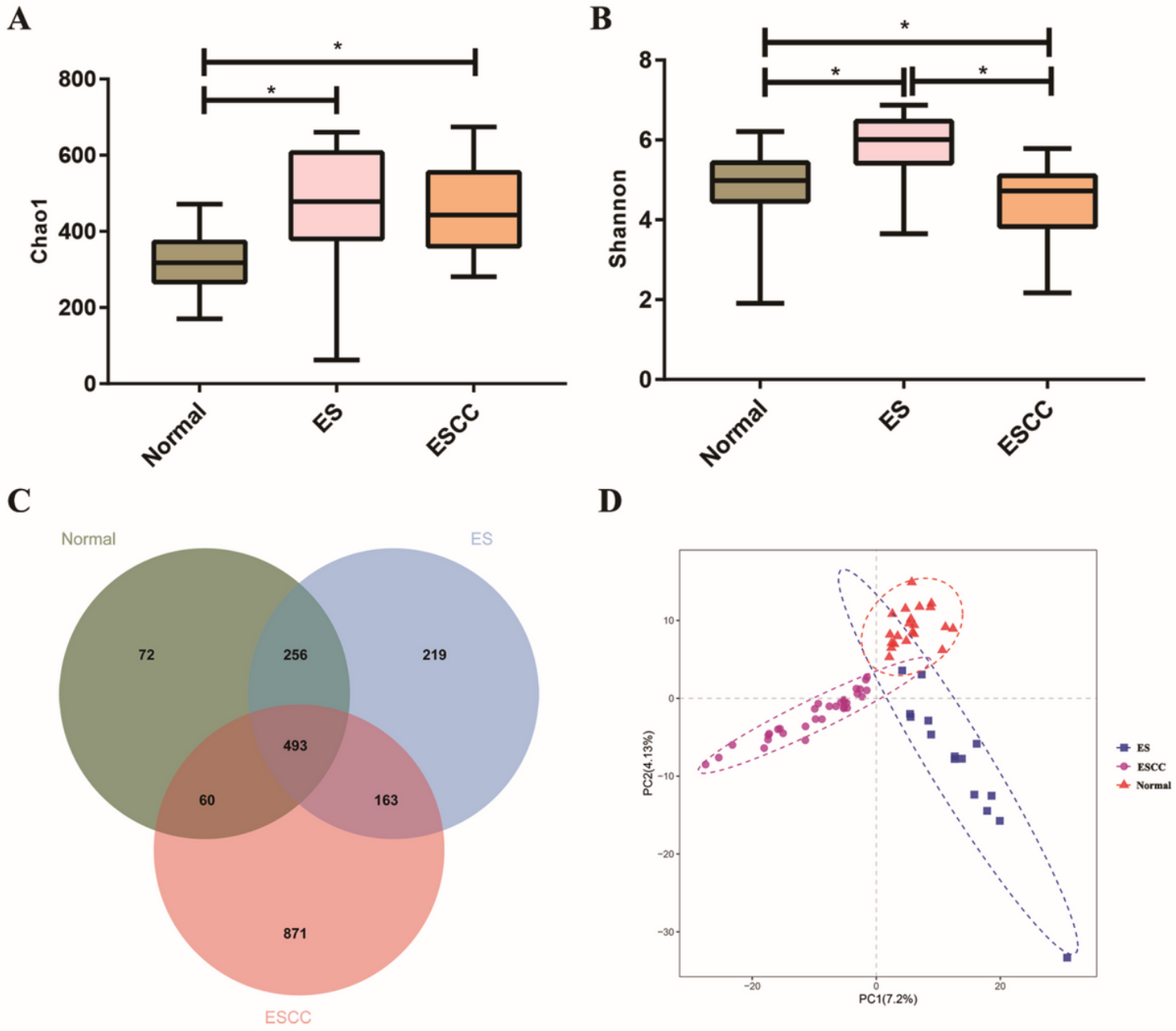

Figure 2

The microbial α diversity and β diversity analysis in different groups. (A) Chao 1 index was higher in ESCC and ES group than in normal group. (B) The ES group had significantly higher Shannon index, in comparison with ESCC group and normal group. (C) A Venn diagram displayed the overlaps among groups. (D) PLS-DA revealed different microbial community structures in the three groups. *, $p<0.05$.

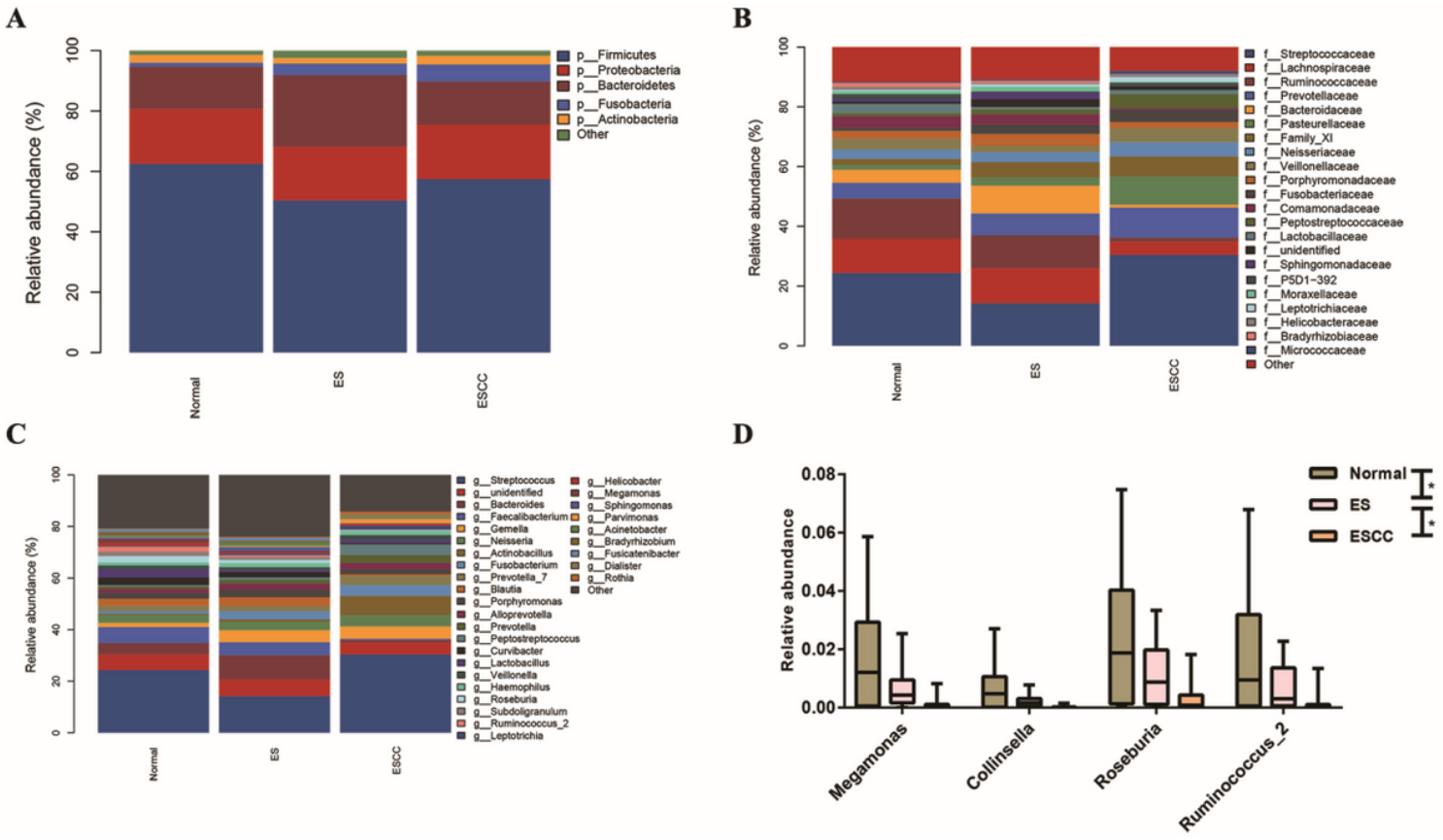

Figure 3

Comparison of relative abundance among each group Barplots of the relative abundance of the main bacterial taxa at (A) phylum, (B) family and (C) genus level for normal, ES and ESCC groups. (D) Mean relative abundance of continuous changing genera among groups. *, p <0.05.

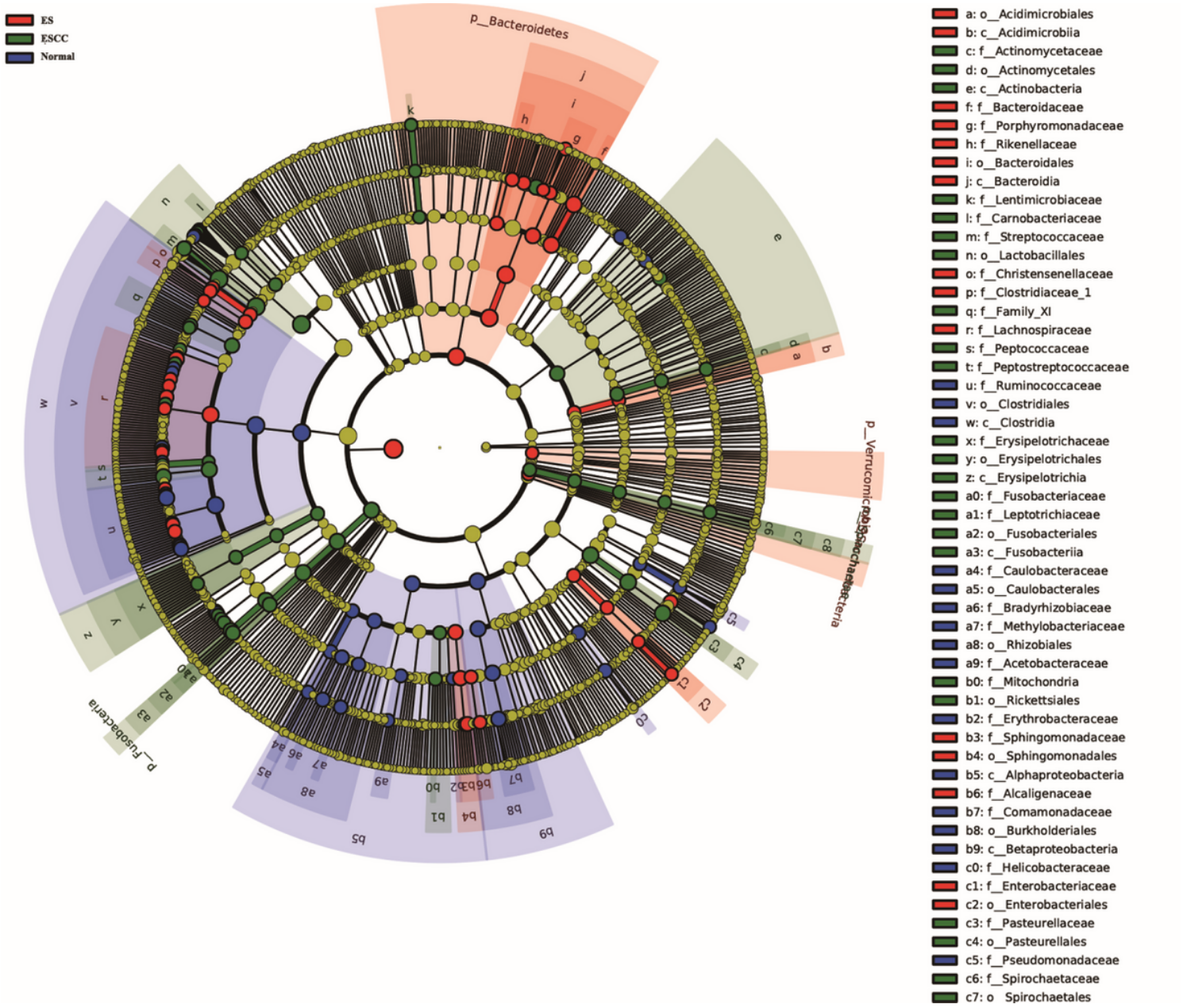

Figure 4

LEfSe analysis showd the most abundant taxa from the phylum to the genus level among normal, ES and ESCC groups.

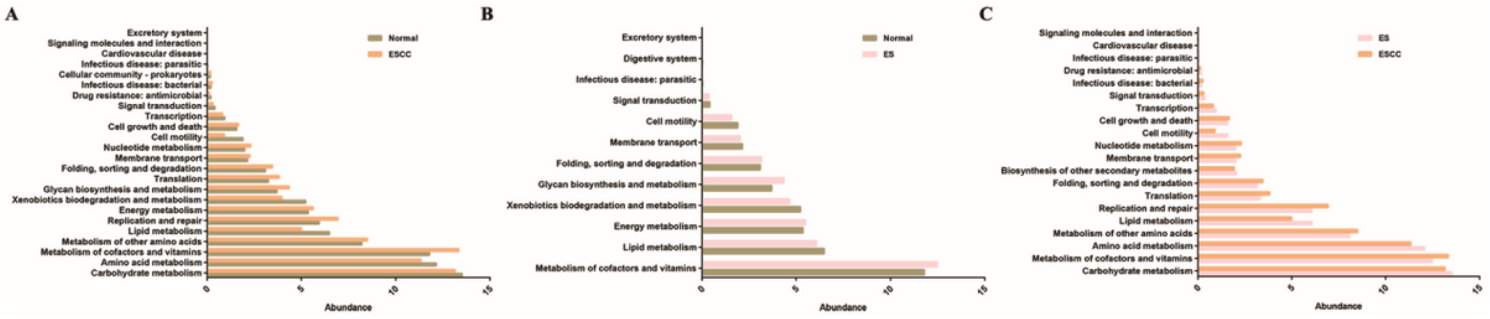

Figure 5

The function prediction of the three groups Differential KEGG pathways were analyzed using PICRUSt for three groups. Significant differences between normal and ESCC group (A), normal and ES group (B), and ES and ESCC group (C) were presented respectively.