International Journal of General Medicine

Dovepress
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ORIGINAL RESEARCH

Alterations in the Gut Microbiota of Patients with Esophageal Squamous Cell Carcinoma: A Cross-Sectional Study Conducted in a High-Incidence Region of China

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Background: Esophageal squamous cell carcinoma (ESCC) is an aggressive and often fatal upper gastrointestinal cancer. Although the gut microbiota plays a critical role in the development of digestive tract malignancies, especially those in the lower gastrointestinal tract, clinical studies exploring its association with ESCC remain limited.

Objective: This study aimed to delineate differences in gut microbiota diversity and composition between ESCC patients and healthy controls (HCs) through full-length 16S rRNA sequencing.

Methods: Between September 2023 and September 2024, 171 fresh fecal samples were obtained from 93 patients with ESCC and 78 HCs in Hebei Province, China. Sequencing of the full-length 16S rRNA gene regions (V1–V9) was conducted using the PacBio Sequel II platform, followed by comprehensive bioinformatic analysis.

Results: Relative to HCs, ESCC patients exhibited markedly lower alpha diversity and distinct beta diversity. At the genus level, enrichment of *Bifidobacterium, Ligilactobacillus, Lactococcus, Intestinibacter, Paucibacter, Acinetobacter*, and *Mogibacterium* was identified in the ESCC cohort. A diagnostic model incorporating these seven genera achieved an area under the curve (AUC) of 0.726 (95% confidence interval: 0.650–0.801). Additionally, these significantly different genera were associated with some clinicodemographic information. Furthermore, a decline in several short-chain fatty acid (SCFA)-producing genera, including *Blautia, Anaerostipes*, and the *Eubacterium_hallii_group*, was observed in ESCC patients. Bacterial phenotype predictions using BugBase and functional profiling with PICRUSt2 further demonstrated significant microbiota differences between patients and healthy subjects.

Conclusion: Comprehensive analysis of the gut microbial community in ESCC patients from Hebei Province, China, revealed significant dysbiosis involving community structure, bacterial phenotypes, functional capacity, and metabolic pathways. These alterations may be potentially associated with the occurrence of ESCC.

Keywords: esophageal squamous cell carcinoma, gut microbiota, gut microbiota dysbiosis, full-length 16S rRNA, SCFA-producing bacteria, cross-sectional study, Hebei province, China

Introduction

In China, ESCC stays the most prevalent histological subtype of esophageal cancer,[1] with Hebei Province reporting particularly high incidence rates.[2–4] Despite notable progress in diagnostic approaches and therapeutic regimens, the five-year survival rate for ESCC continues to fall below 30%.[5] Clarifying the underlying mechanisms of ESCC is therefore imperative, as its pathogenesis is widely recognized to be multifactorial and highly complex.

Received: 27 May 2025
Accepted: 14 October 2025
Published: 27 October 2025

It is widely acknowledged that the development and progression of ESCC involve a multitude of factors; however, the precise mechanisms driving its pathogenesis remain incompletely understood. While numerous studies have focused on external environmental and genetic influences,[6–11] host intrinsic factors also play a critical role in disease pathogenesis, among which the gut microbiota represents a key component of the internal milieu.[12,13]

The gut microbiota is integral to host homeostasis, and dysbiosis has been associated with a range of conditions including obesity, type 2 diabetes, hepatic steatosis, intestinal disorders, and various cancers.[14] With respect to tumorigenesis, extensive evidence supports a link between intestinal microbial dysbiosis and cancer development.[15–18] Disruption of microbial equilibrium has been implicated in metabolic dysregulation, activation of oncogenic signaling, and promotion of ESCC progression.[19] Characteristic microbial alterations in ESCC have been documented, including enrichment of carcinogenic or potentially pathogenic taxa and depletion of beneficial or commensal organisms.[19–21]

Notably, existing studies on the gut microbiota in ESCC are often limited by small sample sizes and reliance on short-read second-generation 16S rRNA sequencing. Larger cohorts would improve the reliability of clinical insights,[22] and third-generation full-length 16S rRNA sequencing offers superior taxonomic resolution for microbial community analysis.[23] With ongoing advancements in PacBio SMRT 16S rRNA sequencing technology, especially the introduction of circular consensus sequencing (CCS), which significantly enhances sequencing accuracy, such improvements have successfully resolved prior doubts about its reliability.[24] Moreover, previous studies have adopted this technology and achieved promising results.[25]

In the present study, we applied PacBio SMRT sequencing to fecal samples collected from 171 individuals in a high-incidence region of China, including 93 patients diagnosed with ESCC and 78 healthy controls. Our overarching aim was to characterize structural and functional metabolic alterations in the gut microbiota associated with ESCC, thereby providing new insights for preventive strategies and personalized therapeutic interventions.

Material and Methods

Research Design

From September 2023 to September 2024, a total of 117 treatment-naïve ESCC patients and 78 HCs were enrolled from the Fourth Hospital of Hebei Medical University. All ESCC diagnoses were histopathologically confirmed and staged according to the 8th edition of the American Joint Committee on Cancer (AJCC) staging system.[26] Healthy volunteers were recruited from the health examination center, and all exhibited test results within normal ranges with no evident abnormalities. From the initial ESCC cohort, 93 patients with stage I–III disease were selected for inclusion in this study, whereas all 78 HCs were included. Demographic information was collected for all participants, encompassing gender, age, body mass index (BMI), smoking behavior history, and alcohol drinking history. For the ESCC group, tumor stage and location were also documented. Written informed consent was secured from every participant.

Participant Inclusion Criteria

The eligibility framework for this study was established based on prior evidence[27] and comprised the following: (1) age between 18 and 85 years; (2) histopathological confirmation of ESCC without any previous diagnosis of malignancy; (3) clinical stage I–IV; and (4) Eastern Cooperative Oncology Group (ECOG) performance status of ≤2. Exclusion criteria included: (1) any prior chemotherapy, radiotherapy, or tumor resection; (2) suspected concurrent malignancies; (3) major cardiovascular events, including myocardial infarction or cerebrovascular accident; (4) administration of probiotics, antibiotics, proton pump inhibitors (PPIs), or hormone-related drugs within two months before enrollment; (5) prior gastrointestinal surgery; (6) history of inflammatory bowel disease (IBD) or irritable bowel syndrome (IBS); and (7) diabetes or depressive disorder. Healthy controls were required to maintain regular bowel habits and to abstain from antibiotics, probiotics, prebiotics, or synthetic agents during the two months preceding sample collection. Furthermore, all participants had to be of Han ethnicity and residents of Hebei Province for a minimum of 10 years.

Collection of Fecal Samples From Participants

Each participant contributed no less than 1.5 g of fecal material, collected in sterile containers, immediately subjected to liquid nitrogen freezing for 10 minutes to preserve microbial viability, and subsequently stored at −80°C until analysis. In total, 171 fresh stool samples were obtained, comprising 93 from individuals with ESCC and 78 from healthy controls.

Bacterial DNA Extraction and Subsequent Sequencing Analysis of the 16S rRNA Gene

Genomic DNA extraction from fecal specimens was executed utilizing the TGuide S96 Magnetic Fecal DNA Kit (TIANGEN Biotech (Beijing) Co., Ltd) in accordance with the manufacturer's instructions. DNA quality verification and concentration quantification were conducted via electrophoresis on a 1.8% agarose gel, while measurements of nucleic acid concentration and assessments of its purity were performed with a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Scientific, Wilmington, USA). The complete 16S rRNA gene sequences underwent amplification with primers 27F: AGRGTTTGATYNTGGCTCAG and 1492R: TASGGHTACCTTGTTASGACTT. Sample-specific PacBio barcode sequences were incorporated in both forward and reverse 16S primers for multiplexed sequencing. The amplification process employed KOD One PCR premix (TOYOBO Life Science) with initial denaturation at 95°C for 2 minutes, succeeded by 25 cycles consisting of denaturation at 98°C for 10 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute 30 seconds, concluding with extension at 72°C for 2 minutes to generate amplified products. Purification of PCR amplicons was achieved using VAHTS TM DNA Clean Beads (Vazyme, Nanjing, China), and quantification utilized the Qubit dsDNA HS Assay Kit with a Qubit 3.0 Fluorometer (Invitrogen, Thermo Fisher Scientific, Oregon, USA). After individual quantification procedures, equivalent amounts of amplicons were combined. The SMRTbell library preparation from amplified DNA utilized the SMRTbell Express Template Prep Kit 2.0. The PacBio Sequel II platform (Beijing Biomarker Technologies Co., Ltd., Beijing, China) performed sequencing of the purified SMRT bell libraries from pooled and barcoded samples using the Sequel II Binding Kit 2.0.

Analysis of Sequencing Data

Bioinformatics analysis was conducted using BMKCloud (http://www.biocloud.net/). Raw sequencing data underwent initial processing with SMRT Link software (v8.0) for quality control and demultiplexing, producing CCS reads. Assignment of sample-specific CCS sequences was performed with Lima (v1.7.0) through barcode recognition. Cutadapt (v2.7) enabled primer detection and sequence filtration, removing CCS reads lacking primers or outside the target length range (1200–1650 bp). Chimeric sequences were excluded using the UCHIME algorithm (v8.1), yielding clean reads. Sequence clustering into operational taxonomic units (OTUs) at 97% similarity was executed with USEARCH (v10.0), discarding OTUs detected fewer than two times across samples. Amplicon sequence variants (ASVs) were generated with DADA2 (v1.20.0), removing variants present in fewer than two occurrences overall. Taxonomic classification of OTUs was performed with the QIIME2 naive Bayesian classifier against the SILVA database (v138.1) using a 70% confidence cutoff. Alpha diversity indices were calculated in QIIME2 (v2020.6) and visualized in R. Beta diversity patterns were examined through principal coordinate analysis (PCoA), comparing community composition with unweighted Jaccard, weighted Bray-Curtis, and weighted UniFrac distances. Group-level differences in beta diversity were statistically evaluated by permutational multivariate analysis of variance (PERMANOVA). Linear Discriminant Analysis (LDA) Effect Size (LEfSe) analysis identified taxa with significant intergroup variation, with a logarithmic LDA score cutoff set at 3.0[19] to emphasize differences in the gut microbiota. BugBase (https://github.com/knights-lab/BugBase) was employed for bacterial phenotype prediction, while PICRUSt2 (https://huttenhower.sph.harvard.edu/picrust) was used to predict microbial community functions, including Cluster of Orthologous Groups of proteins (COG) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.

Statistical Methods

Data were analyzed using SPSS 26.0 (IBM, USA). Demographic traits were contrasted between the ESCC and HC groups: categorical variables were analyzed via the Chi-square test, and continuous variables were examined using the Student's *t*-test. Microbial alpha and beta diversity were assessed by the Student's *t*-test and PERMANOVA, respectively.

Non-parametric tests were employed to analyze differential microbial abundance: the Wilcoxon rank-sum test was used for comparing two groups, and the Kruskal–Wallis test combined with Dunn's post-hoc test was applied for multi-group comparisons. Bacterial phenotypes predicted by BugBase were compared using the Mann–Whitney *U*-test. Predicted COG and KEGG pathway abundances were evaluated using the Student's *t*-test (corrected). Across all analytical procedures, statistical significance was defined as a two-sided p-value < 0.05.

Results

Clinical Features of the ESCC and HCs Groups

All participants were of Han ethnicity from Hebei Province, China. Statistical analyses revealed no notable variations in demographic and clinical baseline parameters between the ESCC and control groups ($p > 0.05$), confirming comparability across cohorts (Table 1).

A Comprehensive Exploration of 16S rRNA Sequencing Outcomes Across All the Participants

From 171 fecal samples, a total of 1,274,715 CCS reads were generated through barcode-based identification, with a per-sample minimum of 4,533 and an average of 7,454 CCS reads. Subsequent taxonomic classification yielded 8,805 OTUs (Supplementary Figure 1), which were annotated to 25 phyla, 763 genera, and 1,960 species. Venn diagram analysis (Figure 1A) identified 6,072 OTUs shared between groups, with 1,238 unique to HCs and 1,495 unique to ESCC patients. Rarefaction curves (Supplementary Figure 2), Shannon index curves (Supplementary Figure 3), and species accumulation curves (Supplementary Figure 4) verified adequate sequencing depth, while rank-abundance distribution curves (Supplementary Figure 5) detailed species richness and evenness across all samples.

Table 1 Below Is a Detailed Account of the Baseline Characteristics for Both the ESCC and HC Groups

Factor	ESCC n(%)	HC n(%)	Statistic	*p* Value
Total Number	93	78		
Age (years)	66.53±6.394	66.13±5.392	t=0.436	0.664
Gender				
Male	70(75.3%)	50(64.1%)	χ^2=2.527	0.112
Female	23(24.7%)	28(35.9%)		
BMI (kg/m^2)	23.74±2.836	23.88±3.761	t=−0.269	0.789
Tobacco smoking				
Yes	56(60.2%)	42(53.8%)	χ^2=0.703	0.402
No	37(39.8%)	36(46.2%)		
Alcohol intake				
Yes	53(57.0%)	45(57.7%)	χ^2=0.009	0.926
No	40(43.0%)	33(42.3%)		
Tumor stage (%)[a]				
I	16(17.2%)	N/A[b]		
II	19(20.4%)	N/A		
III	58(62.3%)	N/A		
Tumor location (%)				
Upper thoracic	26(27.9%)	N/A		
Middle thoracic	45(48.3%)	N/A		
Lower thoracic	22(23.6%)	N/A		

Notes: [a]Staging was performed using the AJCC (8th) staging system; [b]N/A=not applicable; BMI= body mass index.

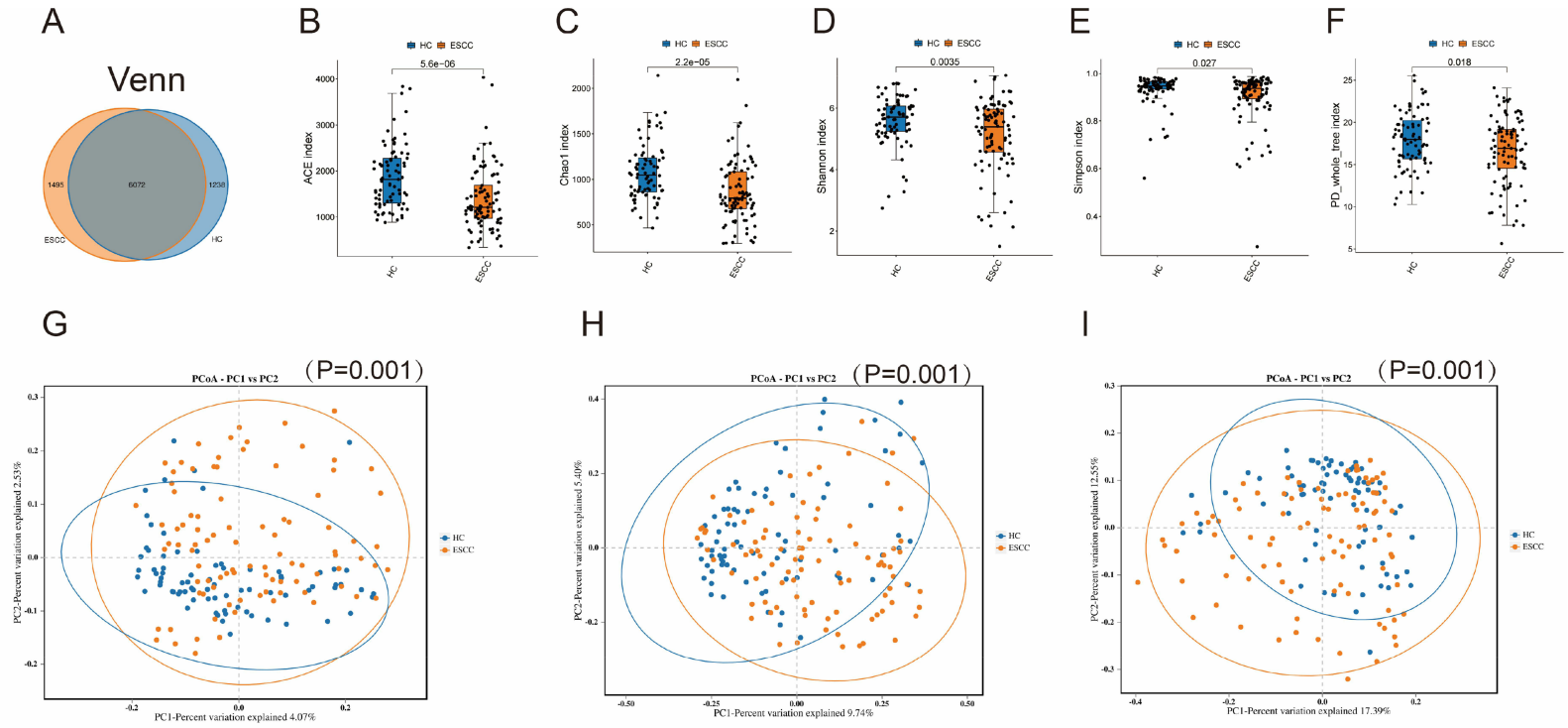

Figure 1 Analysis of OTUs and Diversity Differences Between HCs and ESCC Patients. Venn diagram (**A**) illustrates the shared and distinct characteristics of the intestinal microbiota in two groups. Whereas box plots show the α-diversity indices of the two groups, including ACE index (**B**), Chao 1 index (**C**), Simpson index (**D**), Shannon index (**E**), and PD_whole_tree index (**F**). Statistical evaluations were performed using the Student's *t* test. PCoA plots derived from unweighted Binary Jaccard (**G**), weighted Bray–Curtis (**H**), and weighted UniFrac (**I**) distance matrices were assessed via PERMANOVA testing.

Exploring Gut Microbiota Diversity in ESCC Patients and Healthy Individuals

Alpha diversity analysis (HC group vs ESCC group) showed: ACE index (1885.70±78.14 vs 1390.82±70.69, p = 5.6e-06),Chao 1 index (1080.18±35.45 vs 862.80±35.02, p = 2.2e-05), Shannon index (5.59±0.09 vs 5.13±0.12, p = 0.0035), Simpson index (0.93±0.01 vs 0.90±0.01, p = 0.027), and PD_whole_tree index (17.92±0.38 vs 16.57±0.42, p = 0.018) (Figure 1B–F). PCoA plots of unweighted Jaccard, weighted Bray–Curtis, and weighted UniFrac uncovered the distinct features of the two groups of samples (Figure 1G–I). PERMANOVA tests for unweighted binary Jaccard (R^2=0.011, p=0.001), weighted Bray–Curtis (R^2=0.021, p=0.001), and weighted UniFrac (R^2=0.028, p=0.001) demonstrated variations that are statistically significant in beta diversity between the two groups.

Analysis of the Intestinal Microbiota Composition Between the ESCC and HCs Groups

Phylum-level profiling revealed *Firmicutes* as the most abundant phylum in both groups, representing 84.99% in HCs and 71.73% in ESCC. The five predominant bacterial phyla were consistent between the two groups (*Firmicutes, Bacteroidota, Proteobacteria, Actinobacteriota, Verrucomicrobiota*), but significant changes in relative abundances were observed. Compared with HCs, the ESCC cohort displayed higher proportions of *Bacteroidota* (11.82% vs 8.21%), *Proteobacteria* (9.37% vs 3.84%), *Actinobacteriota* (4.65% vs 1.78%), and *Verrucomicrobiota* (0.98% vs 0.23%), while *Firmicutes* was relatively enriched in HCs (84.99% vs 71.73%) (Figure 2A).

At the genus level, *Blautia* predominated in both cohorts (16.85% in HCs vs 12.74% in ESCC). The ESCC group showed increased representation of *Bacteroides* (8.82% vs 6.11%), *Ruminococcus* (3.27% vs 2.42%), *Escherichia_Shigella* (3.80% vs 1.40%), and *Streptococcus* (2.94% vs 2.25%), whereas HCs were characterized by higher levels of *Blautia* (16.85%), *Faecalibacterium* (4.53%), *Romboutsia* (6.16%), *Eubacterium_hallii_group* (5.96%), *Dorea* (4.26%), and *Agathobacter* (4.02%) (Figure 2B).

Species-level comparison identified *Romboutsia_timonensis* as most abundant in HCs (6.02%) and *Eubacterium_hallii* in ESCC (4.26%). *Escherichia_coli* was notably increased in ESCC (3.80% vs 1.39%), while HCs exhibited enrichment of *Faecalibacterium_prausnitzii* (5.27%), *Romboutsia_timonensis* (6.02%), *Eubacterium_hallii* (5.92%), *Eubacterium_rectale* (3.82%), *Lachnospiraceae_bacterium* (3.28%), *Dorea_longicatena* (3.44%), and multiple *Blautia* subspecies, including *B. obeum* (4.08%), *B. massiliensis* (2.68%), and *B. wexlerae* (3.21%) (Figure 2C).

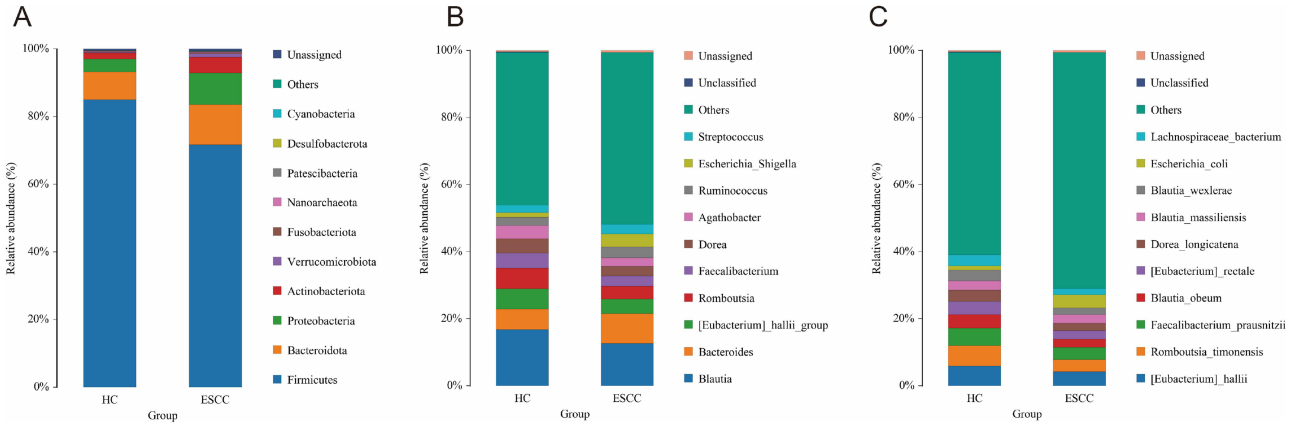

Figure 2 Composition Distribution of Intestinal Microbiota at Multiple Taxonomic Levels Between the HC Group and the ESCC Group. Bar charts illustrate the top 10 most abundant phyla (**A**), genera (**B**), species (**C**) in the gut microbiota of both groups. Different colors represent distinct species, with the vertical extent of each colored segment indicating the relative abundance of that species. Species outside the top 10 are grouped as "Others", unannotated taxa are classified as "Unknown", and unclassified taxa are marked as "Unassigned".

Differential Microbiota Analysis Between the ESCC and HCs Groups

Comparative microbial community structures were evaluated using Wilcoxon rank-sum tests across multiple taxonomic levels.

At the phylum level, 8 phyla displayed significant intergroup differences. *Firmicutes* ($p = 0.0004$) showed higher relative abundance in HCs, whereas *Actinobacteriota* ($p = 0.0004$) and *Proteobacteria* ($p = 0.0290$) were significantly elevated in ESCC (Figure 3A).

At the genus level, 155 genera differed markedly between groups. *Blautia* ($p = 0.0021$), *Eubacterium_hallii_group* ($p = 0.0002$), *Faecalibacterium* ($p = 0.0021$), *Dorea* ($p = 0.0019$), and *Agathobacter* ($p = 0.0005$) were enriched in HCs, while *Streptococcus* ($p = 0.0368$) predominated in ESCC (Figure 3B).

At the species level, 331 species exhibited significant divergence. *Blautia_massiliensis* (p = 0.0035), *Lachnospiraceae_bacterium* ($p = 1.22e-05$), *Eubacterium_hallii* ($p = 0.0002$), *Eubacterium_rectale* ($p = 0.0002$), *Blautia_obeum* ($p = 0.0003$), *Blautia_wexlerae* ($p = 0.0007$), *Dorea_longicatena* ($p = 0.0021$), and *Faecalibacterium_prausnitzii* ($p = 0.0044$) showed enrichment in HCs, whereas Streptococcus_thermophilus ($p = 0.0013$) was significantly more abundant in ESCC (Figure 3C).

LEfSe Analysis Reveals Significantly Enriched Differential Intestinal Microbiota Between ESCC Group and HCs

Analysis of the intestinal microbiota associated with ESCC was performed using LEfSe with an LDA threshold >3 to identify taxa displaying significant differential enrichment between the ESCC and HC groups (Figure 4). A total of 23 taxa demonstrated higher abundance in the ESCC group, whereas 54 taxa predominated in the HCs. At the genus level, ranked by descending LDA scores, *Bifidobacterium, Ligilactobacillus, Lactococcus, Intestinibacter, Paucibacter, Acinetobacter*, and *Mogibacterium* were significantly enriched in ESCC (LDA > 3). In contrast, enrichment in HCs was observed for *Blautia, Holdemanella, Eubacterium_hallii_group, Anaerostipes, Dialister, Ruminococcus_torques_group, Faecalibacterium, Agathobacter, Dorea, Subdoligranulum*, and *Erysipelotrichaceae_UCG_003* (LDA > 3.5).

Potential Biomarkers for Distinguishing ESCC Patients From Healthy Subjects

LEfSe and LDA analyses revealed seven genera with significant enrichment in the gut microbiota of ESCC patients, with *Bifidobacterium* displaying the highest LDA score. Receiver Operating Characteristic (ROC) curve analysis for this genus yielded an AUC of 0.64 (95% CI: 0.556–0.723) (Supplementary Figure 6). To enhance diagnostic accuracy, a composite model was constructed by integrating all seven genera, which produced an improved AUC of 0.726 (95% CI: 0.650–0.801), indicating its potential value in distinguishing ESCC patients from healthy individuals (Figure 5).

Associations Between Microbial Taxa and Clinicodemographic Features in ESCC

Associations between microbial taxa and clinicodemographic features were further evaluated in patients with ESCC. Significant variations in microbial abundance were observed according to clinical stage: *Acinetobacter* levels differed between HCs and patients with stage T1 ($p = 0.0070$) or T3 ($p = 0.0250$) ESCC; *Bifidobacterium* abundance varied between HCs and T1 ($p = 0.0090$) or T3 ($p = 0.0160$) stages; and *Mogibacterium* was significantly different specifically between HCs and T2-stage ESCC ($p = 0.0020$) (Supplementary Table 1).

Tumor location was also a determining factor: *Bifidobacterium* was enriched in the middle thoracic segment of ESCC tumors relative to HCs ($p = 0.0001$) and the lower thoracic segment ($p = 0.0240$). *Intestinibacter* was more abundant in ESCC upper thoracic segments compared to HCs ($p = 0.0004$), lower thoracic segments ($p = 0.0030$), and middle thoracic segments ($p = 0.0150$). *Ligilactobacillus* was elevated in ESCC upper thoracic segments relative to both HCs ($p = 0.0060$) and lower thoracic segments ($p = 0.0110$), while *Mogibacterium* was higher in the middle thoracic segment than in HCs ($p = 0.0080$). Conversely, *Paucibacter* was reduced in ESCC lower thoracic segments compared to HCs ($p = 0.0030$) (Supplementary Table 2).

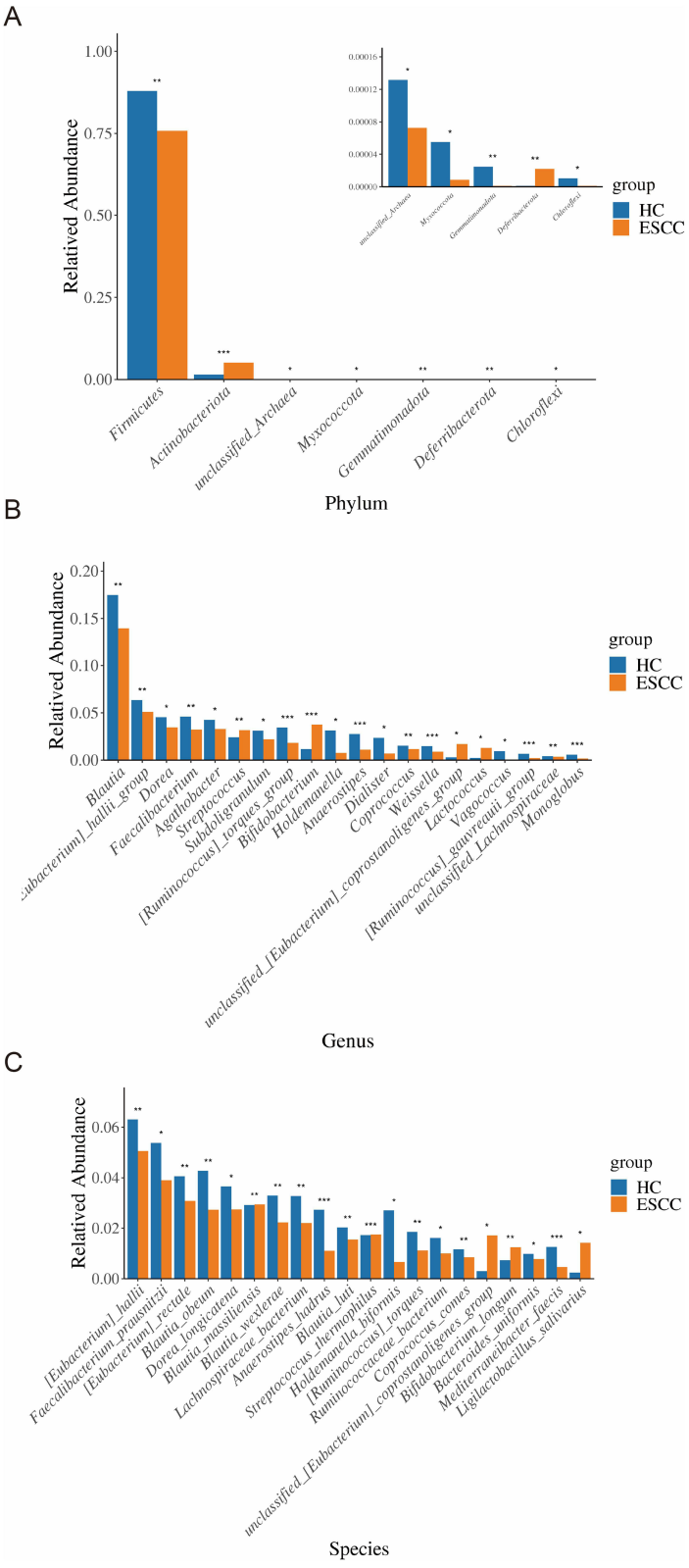

Figure 3 Comparison of Intestinal Microbiota Between HCs and ESCC Patients at Different Taxonomic Levels. All notable variations at the phylum level (**A**) are displayed, and the top 20 intestinal bacteria exhibiting differences at the genus (**B**) and species (**C**) levels are presented (Wilcoxon rank test, $p < 0.05$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

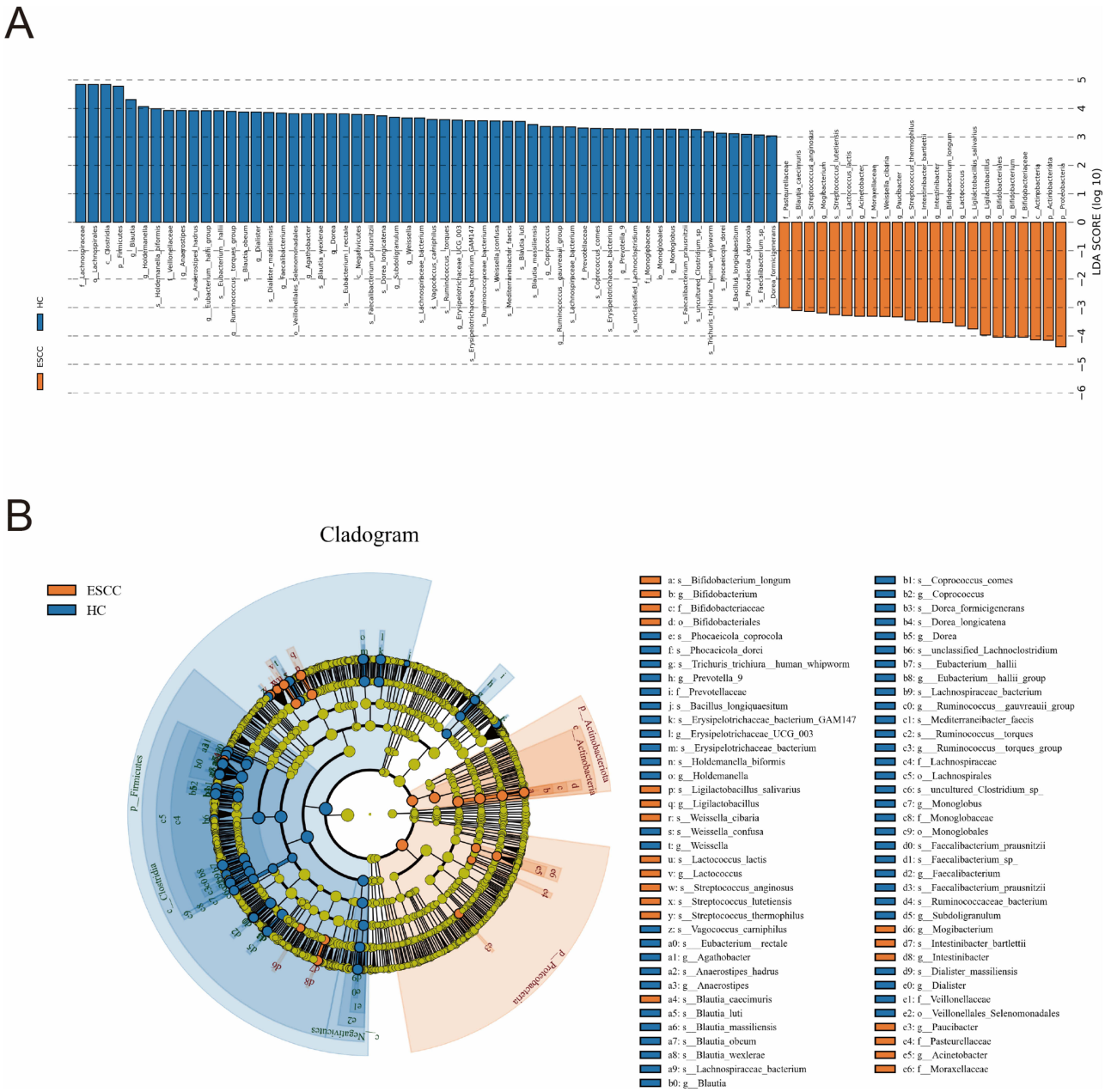

Figure 4 LEfSe Application for Detecting Taxonomic Groups with Significant Abundance Differences in Gut Microbiota of HC and ESCC Patients (Wilcoxon rank sum test, $p < 0.05$). The LDA histogram (**A**) illustrates microbial classifications exhibiting LDA scores greater than 3 within the intestinal microbiota of both groups. The phylogenetic tree of bacterial indicators (**B**) ranging from phylum (the innermost ring) to species (the outermost ring), emphasizing microbial taxa that exhibit an LDA score exceeding 3. Unique bacterial groups are marked with lowercase letters. At each taxonomic level, every small circle stands for a single taxonomic unit, and the circle's size is proportional to its relative abundance. Colors distinguish between the HC and ESCC groups, while nodes of different colors indicate key microbial communities specific to each group.

Sex-specific differences were also observed: *Intestinibacter* was higher in female than male ESCC patients ($p = 0.0002$). Compared to female HCs, ESCC females had elevated *Bifidobacterium* ($p = 0.0480$), while ESCC males showed reduced *Lactococcus* relative to male HCs ($p = 0.0040$). Older ESCC patients exhibited higher *Intestinibacter* levels than younger patients ($p = 0.021$). Low-BMI ESCC patients had more *Bifidobacterium* ($p = 0.005$) and *Intestinibacter* ($p = 0.006$) than low-BMI HCs (Supplementary Tables 3–5).

Lifestyle factors influenced microbial abundance: non-smoking ESCC patients had higher *Bifidobacterium* and *Intestinibacter* than smoking HCs ($p = 0.0003$ and $p = 0.0009$, respectively). Similarly, non-drinking ESCC patients showed elevated levels of these genera compared to drinking HCs ($p = 0.0030$ and $p = 0.0008$) (Supplementary Tables 6 and 7).

https://doi.org/10.2147/IJGM.S541736

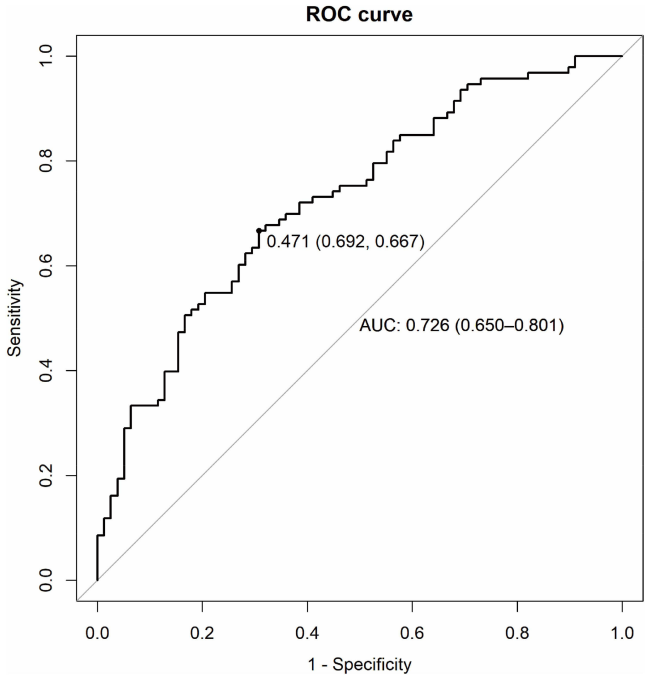

Figure 5 ROC Curve for Significantly Enriched Bacterial Genera in the Gut Microbiota of Patients Diagnosed with ESCC. This diagnostic model includes seven bacterial genera, namely *Bifidobacterium, Ligilactobacillus, Lactococcus, Intestinibacter, Paucibacter, Acinetobacter*, and *Mogibacterium*. The AUC was 0.726 (95% CI: 0.650–0.801), with a cutoff value of 0.471 (Sensitivity = 0.692, Specificity = 0.667).

Prediction of Gut Microbiota Functions in ESCC and HC Groups

BugBase-based microbial phenotype prediction was applied to delineate the functional attributes of microbial communities and their relevance to health and disease.[28] Relative to the HC group, the ESCC group exhibited a marked increase in facultative anaerobes ($p = 0.0046$, Figure 6A) and gram-negative bacteria ($p = 0.0066$, Figure 6B), accompanied by a reduction in anaerobic bacteria ($p = 0.0009$, Figure 6C) and gram-positive bacteria ($p = 0.0066$, Figure 6D). Functional inference from 16S rRNA data was subsequently conducted using PICRUSt2. COG annotation indicated significant enrichment in the ESCC group for categories including "Posttranslational modification, protein turnover, chaperones", "Intracellular trafficking, secretion, and vesicular transport", "Inorganic ion transport and metabolism", "Cell wall/membrane/envelope biogenesis", "Secondary metabolites biosynthesis, transport and catabolism", "RNA processing and modification", "Lipid transport and metabolism", "Function unknown", "Extracellular structures", and "Chromatin structure and dynamics", whereas "Transcription", "Signal transduction mechanisms", "Defense mechanisms", and "Cell cycle control, cell division, chromosome partitioning" were diminished (Figure 6E). KEGG annotation further demonstrated significant enrichment in the ESCC group for pathways related to "digestive system", "other amino acid metabolism", "cancer: overview", "endocrine system", "infectious diseases: parasitic diseases", "aging", "transport and catabolism", "antitumor drug resistance", "biodegradation and metabolism of xenobiotics", "glycan biosynthesis and metabolism", "bacterial infectious diseases", and "lipid metabolism", while activity in "cofactor and vitamin metabolism" was significantly reduced (Figure 6F).

Discussion

In this study, full-length 16S rRNA sequencing identified marked alterations in the gut microbiota of ESCC patients relative to healthy controls, including both α- and β-diversity as well as enrichment of discriminatory bacterial genera determined through LEfSe. A diagnostic model constructed from these taxa exhibited potential diagnostic value. Furthermore, the abundance of some of these discriminative genera was significantly associated with clinicodemographic characteristics, suggesting host-microbe interactions may be influenced by patient demographics and disease status. Functional predictions via BugBase and PICRUSt2 suggested that these microbial shifts may be linked to altered

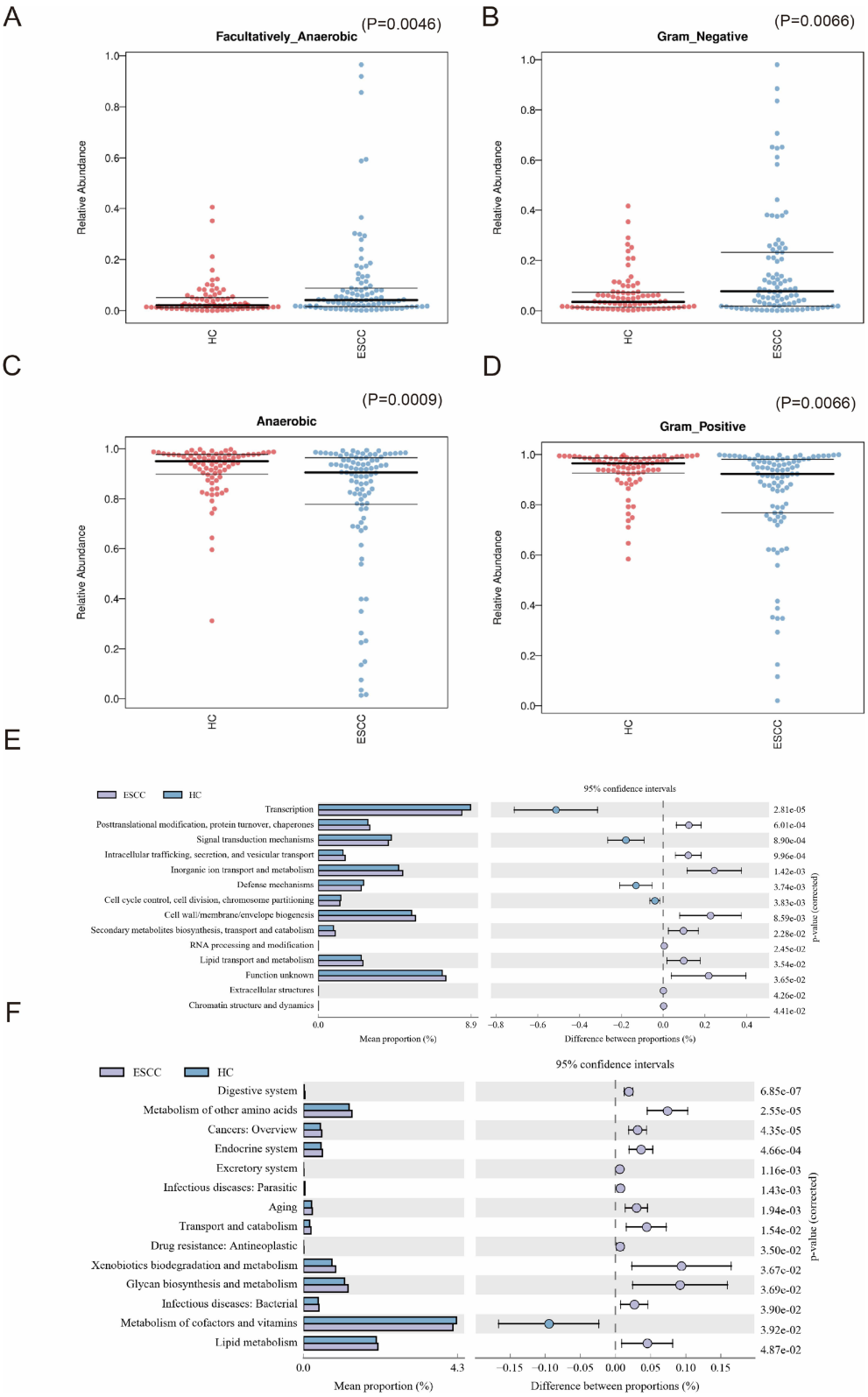

Figure 6 Phenotypic and Functional Prediction Analysis of the Gut Microbiome in the HC Group and the ESCC Group. Scatter plots illustrate the phenotypes of facultative anaerobes (**A**), gram-negative bacteria (**B**), anaerobic bacteria (**C**) and gram-positive bacteria (**D**) in each group, with significance statistically evaluated by the Mann–Whitney *U*-test. The disparities in COG function (**E**) and the predictive analysis of the second-level metabolic pathways of the KEGG (**F**) between the two groups were analyzed using Student's *t* test, corrected $p < 0.05$.

https://doi.org/10.2147/IJGM.S541736

bacterial traits and metabolic pathways. Collectively, these results may provide biological insights that could inform the development of microbiota-based screening and therapeutic approaches for ESCC, and could help advance our understanding of host–microbiota interactions in cancer.

Analysis of microbial diversity demonstrated a significant reduction in alpha diversity among ESCC patients compared with controls, in agreement with prior findings by Shen et al.[29] Variability in reported alpha diversity across studies may reflect heterogeneity in study populations, sequencing platforms, or potential confounders such as pharmacological exposure.[19–21] By contrast, alterations in beta diversity have been consistently documented,[19–21,28–30] suggesting that restructured microbial community composition, rather than simple richness or evenness, constitutes a more reliable feature of ESCC and may capture signals associated with tumor burden.

Analysis of taxonomic composition emphasized alterations at the genus level. Unlike several earlier reports, significant enrichment of pathogen-associated genera frequently described in ESCC, including *Streptococcus*,[19] *Bacteroides*[21] and *Escherichia_Shigella*[28] was not detected. In contrast, enrichment of *Bifidobacterium* aligned with previous observations.[30] Importantly, this investigation provides, to the best of current knowledge, the first evidence of substantial enrichment of additional genera with pathogenic potential in ESCC, such as *Intestinibacter, Acinetobacter*, and *Mogibacterium*. These newly identified microbial features may be closely linked to region-specific factors: our samples were from Hebei Province (northern China), where high dietary sodium intake and arid climate are prevalent, and these conditions likely shape the distinctive gut microbiota composition in this population. Consistent with this, previous studies[31] have explicitly shown that dietary and geographical differences have a significant, observable impact on gut microbiota.

Furthermore, our analysis showed that the abundance of these discriminative genera had heterogeneous patterns but varied significantly with clinicodemographic features, often in non-linear relationships. For instance, *Acinetobacter* and *Bifidobacterium* relative abundances did not increase continuously from normal controls to T3-stage ESCC patients. Patients with middle/lower thoracic tumors exhibited a significantly lower relative abundance of *Intestinibacter* than did patients with upper thoracic tumors, suggesting a specific association with tumor longitudinal localization. Beyond tumor stage and location, variables including sex, age, BMI, smoking, and alcohol consumption were also linked to specific microbial differences, though the clinical implications of these associations require further assessment. These nuanced associations support the premise that gut microbiota is closely tied to ESCC's clinicopathological landscape.[21]

Further analysis demonstrated significant enrichment of *Bifidobacterium* in ESCC patients' gut microbiota. Although consistent with previous observations,[30] this pattern presents a paradox regarding the functional implications of *Bifidobacterium*. Traditionally *Bifidobacterium* is regarded as a probiotic with health-promoting effects linked to bile acid metabolism.[32,33] Yet, earlier studies have demonstrated that bile acids may act as key mediators in esophageal carcinogenesis.[34,35] Thus, we must interpret this seemingly contradictory phenomenon with extreme caution.[36] Moreover, *Bifidobacterium* generates metabolites such as lactate and acetate.[37] Lactate accumulation has been associated with protumorigenic activities, including angiogenesis, migration, and metastasis.[38] A colorectal cancer study from Italy that integrated microbiome and metabolomic profiling reported a positive association between *Bifidobacterium* abundance and lactate levels,[39] emphasizing the necessity of validating this relationship in ESCC through metabolomic approaches. Given that acetate has been demonstrated to act as an energy source for tumor cells,[40] the hypothesis that microbiota-derived acetate functions similarly as an energy source for ESCC cells further warrants targeted metabolomic investigation. In addition, mounting preclinical and clinical evidence has linked *Bifidobacterium* to responsiveness to immune checkpoint therapy (ICT), including treatment with anti-PD-1/PD-L1 antibodies across multiple cancer types.[41–43] This emerging association reflects the intricate interplay between the gut microbiota and antitumor immunity and suggests significant translational and clinical potential for exploiting microbiome modulation in cancer therapeutics.

Beyond *Bifidobacterium*, other differentially enriched genera in our study also warrant mechanistic scrutiny, particularly those with known pathogenic traits. *Acinetobacter*, a genus of pathogenic bacteria characterized by complex and broad-spectrum antibiotic resistance,[44] can evade host innate immune defenses, proliferate rapidly to high biomass, and trigger significant inflammatory responses.[44,45] Emerging evidence further suggests its potential carcinogenicity: it has been reported to form biofilms on human epithelial surfaces, facilitating attachment, colonization, and infection.[46] Biofilms are thought to promote tumorigenesis through multiple mechanisms, including inflammation-mediated DNA

damage, modulation of host immunity, production of carcinogenic toxins, alterations to local metabolic environments, and interactions with other microorganisms in the tumor microenvironment.[47] These features imply that *Acinetobacter* may adversely affect ESCC progression, justifying further in-depth studies into its clinical relevance and mechanistic roles. The esophageal mucosal biofilm may serve as a key mediator in gut microbiota-driven regulation of ESCC. As reviewed by Song et al[48] the esophageal biofilm is not a simple microbial aggregate but a functional complex: its dysbiosis can directly promote carcinogenesis by secreting toxins to activate the NF-κB pathway and producing metabolites such as lactate to induce epithelial cell proliferation. Concurrently, gut pathogens may colonize the esophagus retrogradely via gastroesophageal reflux, participate in biofilm formation, and thereby establish a "gut-biofilm-tumor" regulatory axis.[48]

In the present study, LEfSe analyses identified seven bacterial genera with significant differential abundance: *Bifidobacterium, Ligilactobacillus, Lactococcus, Intestinibacter, Paucibacter, Acinetobacter*, and *Mogibacterium*, which represent potential microbial biomarkers to distinguish ESCC patients from HCs. A diagnostic model built using these candidate biomarkers exhibited good discriminatory performance (AUC = 0.726, 95% CI: 0.650–0.801), directly validating their diagnostic utility and supporting the model's potential as an auxiliary ESCC screening tool. Overall, our work represents a beneficial preliminary step toward non-invasive diagnosis for patients with ESCC, and further external validation is needed to enhance the potential value of the diagnostic model.[49]

Beyond diagnostic relevance, the observed microbial alterations may indicate functional disruptions within the gut ecosystem. Notably, ESCC patients exhibited marked depletion of several SCFA-producing genera, including *Blautia, Eubacterium_hallii group, Anaerostipes, Ruminococcus torques group*, and *Faecalibacterium*, in comparison with HCs. SCFAs such as acetate, propionate, and butyrate, generated through microbial fermentation,[50] serve as central metabolites influencing host cell differentiation and apoptosis, regulating immune responses, and sustaining intestinal barrier integrity.[51] Evidence consistently demonstrates that SCFAs, particularly butyrate, exert anti-inflammatory, barrier-protective, and anti-neoplastic effects.[52–54] Accordingly, the depletion of SCFA-producing taxa in ESCC patients may correspond to reduced SCFA availability, thereby weakening their protective influence on the host. As this conclusion remains preliminary, subsequent studies incorporating serological or fecal metabolomic analyses will be required to establish whether gut microbiota-derived SCFA levels are significantly altered in ESCC.

Predictive functional profiling revealed a markedly altered gut microbiota in ESCC patients, characterized by enrichment of gram-negative bacteria and facultative anaerobes, with concomitant upregulation of pathways related to membrane biogenesis, intracellular transport, and metabolic reprogramming. Such alterations indicate a microenvironment conducive to tumor progression.[55] Nevertheless, as these results are based on in silico predictions, metabolomic and mechanistic validation is needed to confirm their relevance to ESCC pathogenesis.

Future research will adopt a multicenter framework to overcome the restricted generalizability of single-region recruitment, apply stringent control of confounding variables such as diet and lifestyle, and incorporate longitudinal sampling across disease stages. It will also integrate comprehensive documentation of dietary habits, lifestyle, demographics, and extended clinical indicators (beyond tumor stage/location). Subsequent analyses will examine associations between differentially enriched genera (eg, *Acinetobacter, Bifidobacterium*) and additional clinical parameters (eg, age, sex, comorbidities) to identify microbiota-clinical trait correlations with prognostic relevance. This optimized design is anticipated to generate more broadly applicable insights into the interactions between gut microbiota and ESCC and to support the translation of microbial biomarkers into clinical practice.

Several limitations should be acknowledged: (1) A cross-sectional, single-center design compromises sample representativeness; multicenter studies are needed to validate findings in diverse populations. (2) Results indicate correlation, not causality—mechanistic validation (eg, animal studies) and clinical-basic research integration are required. (3) Inclusion of only treatment-naïve, stage I–III ESCC patients excludes advanced cases, limiting insight into dynamic microbiota changes during progression; future studies should enroll patients across all stages/treatment statuses.

Conclusion

Our findings demonstrate that ESCC patients exhibit significant gut microbiota dysbiosis in structure, composition, and function. Among the significantly differential bacterial genera identified by LEfSe, these taxa not only hold potential diagnostic value, but some also exhibit differential abundance patterns that correlate with clinicodemographic characteristics. These specific microbial perturbations provide new insights into ESCC etiology and reinforce its close link to ESCC development. This study lays a preliminary theoretical foundation for the future development of microbiota-based strategies for ESCC prevention or adjuvant therapy.

Data Sharing Statement

All original data sequences are accessible through the Sequence Read Archive (SRA) database at the National Center for Biotechnology Information (NCBI). The accession number is PRJNA1255730 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1255730). The raw data for the samples involved in this study are presented in Supplementary Table 8.

Ethical Statement

This investigation represents a prospective descriptive investigation carried out per the Declaration of Helsinki and sanctioned by the Ethics Committee of the Fourth Hospital of Hebei Medical University (2022KY057). All participants gave written informed consent and agreed to participate in the study.

Acknowledgments

The authors thank Shanghai Biotree Biomedical Technology Co., Ltd for its assistance in sample testing for all subjects in this study.

Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work.

Funding

This research was supported by the National Natural Science Foundation of China (Project No. 82274593), the S&T Program of Hebei (Project No. 223777122D), the Natural Science Foundation Project of Hebei Province (Project No. H2023206137), and the Administration of Traditional Chinese Medicine of Hebei Province (Project No. 2024283).

Disclosure

The authors state that they have no competing interests in this work.

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