Original research article

Gut microbial dysbiosis and its association with esophageal cancer

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Abstract

Due to its aggressive nature and low survival rate, esophageal cancer is one of the deadliest cancer. While the intestinal microbiome significantly influences human health and disease. This research aimed to investigate and characterize the relative abundance of intestinal bacterial composition in esophageal cancer patients. The fecal samples were collected from esophageal cancer patients ($n = 15$) and healthy volunteers ($n = 10$). The PCR-DGGE was carried out by focusing on the V3 region of the 16S rRNA gene, and qPCR was performed for *Bacteroides vulgatus*, *Escherichia coli*, *Bifidobacterium*, *Clostridium leptum* and *Lactobacillus*. High-throughput sequencing of the 16S rRNA gene targeting the V3+V4 region was performed on 20 randomly selected samples. PCR-DGGE and High-throughput diversity results showed a significant alteration of gut bacterial composition between the experimental and control groups, which indicates the gut microbial dysbiosis in esophageal cancer patients. At the phylum level, there was significant enrichment of Bacteroidetes, while a non-significant decrease of Firmicutes in the experimental group. At family statistics, a significantly higher level of Bacteroidaceae and Enterobacteriaceae, while a significantly lower abundance of Prevotellaceae and Veillonellaceae were observed. There was a significantly high prevalence of genera *Bacteroides*, *Escherichia-Shigella*, while a significantly lower abundance of *Prevotella_9* and *Dialister* in the experimental group as compared to the control group. Furthermore, the species analysis also showed significantly raised level of *Bacteroides vulgatus* and *Escherichia coli* in the experimental group. These findings revealed a significant gut microbial dysbiosis in esophageal cancer patients. So, the current study can be used for the understanding of esophageal cancer treatment, disease pathway, mechanism, and probiotic development.

Keywords: DGGE; Esophageal cancer; High-throughput sequencing; Intestinal dysbiosis; Microbiome

Highlights:

- The gut microbial dysbiosis was observed in esophageal cancer patients
- A significant enrichment of Bacteroidetes, but non-significant decrease in Firmicutes was observed in esophageal cancer patients.
- Family statistics showed significant high levels of Bacteroidaceae and Enterobacteriaceae in esophageal cancer patients.
- The high abundant genera: *Bacteroides*, *Escherichia-Shigella*; lower abundance genera: *Prevotella_9* and *Dialister*.
- The species analysis found a significant increase of *Bacteroides vulgatus* and *Escherichia coli* in esophageal cancer patients.

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Introduction

Human intestinal flora is a significant health determinant, as the microorganisms perform metabolic, trophic, and protective functions to protect the body from many diseases (Guarner and Malagelada, 2003). Different factors such as age, diet, and disease could affect their composition and function (Power et al., 2013). An average individual contains approximately a hundred trillion gut microorganisms, important in host homeostasis, i.e., immunity, nutrition, metabolism and absorption (Walsh et al., 2014). Alteration in the gut microbial composition can cause various diseases such as type II diabetes, viral diarrhea, Crohn's disease, colitis, inflammatory bowel disease, Grave's disease, smoking, and asthma (Ishaq et al., 2017b; 2018a, b). Cancer is a leading cause of death worldwide (Hameed et al., 2017; Mohammad et al., 2018a, b; 2020a, b). Malignancy like esophageal cancer is one of the lethal and aggressive disorders, increased dramatically in the last few decades. Due to the lack of early signs and symptoms, most esophageal cancer cases are diagnosed in its advanced stages; therefore, it only hits a survival rate of 5 years (Huang and Yu, 2018). It can be divided into two predominant histological categories (i) squamous cell carcinoma and (ii) adenocarcinoma (Triboulet and Mariette, 2008). The adenocarcinoma has been observed in the distal part of the esophagus that dominates in the Western part of the globe. However, squamous cell cancer grows in the middle part of the thoracic esophagus and prevails in the Eastern part of the world. Squamous cell carcinoma and adenocarcinoma can be differentiated on the basis of molecular characteristics such as squamous cell carcinomas frequently exhibited genomic augmentations of SOX2, CCND1, or TP63, while VEGFA, ERBB2, GATA6 and GATA4 commonly amplified in adenocarcinoma (The Cancer Genome Atlas Research Network, 2017). Therefore, multimodal therapeutic strategies, including chemotherapy and surgery have

been employed to treat this, but the prognosis is still very poor (Baba et al., 2014). However, the gut bacteria compete for nutrients and produce the bacteriocins, protecting the intestine lining from colonization (Guarner and Malagelada, 2003). In line with this, alteration in gut microbial composition could lead to various types of cancers including intestine, stomach, breast or lung. To date, the link between intestinal bacteria and esophageal cancer has not been explored. Thus, current study was designed to elucidate the overall composition along with variation in intestinal bacteria in esophageal cancer patients. PCR-DGGE, qPCR and highly sensitive metagenomics Illumina based Hiseq2500 High-throughput sequencing was used to monitor the gut microbial diversities and similarities in esophageal cancer patients in comparison with healthy control. The current study proves a significant disparity of bacterial community richness between the two groups, with certain intestinal microbes illustrating significantly lower or higher abundance, as illustrated in Fig. 1.

Material and methods

Ethical statement

The study protocol was approved by the School of Medicine, Xi'an Jiaotong University Ethics Committee. Furthermore, all procedures performed in this study were by the laws and rules of the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent: Written informed consent was obtained from all individual participants included in the study.

Collection of fecal samples

A total of 15 stool samples were collected from both squamous cell carcinoma and adenocarcinoma esophageal cancer patients (10 males and 5 females, 45–60 year age) and 10 from healthy individuals (6 males and 4 females, 45–60 years age) by using

Fig. 1. Gut microbial dysbiosis in esophageal cancer patients

a sterile cup. The type and grade of esophageal cancer patients are described in Suppl. Table 1. Each participant's questionnaire was filled containing information on gender, health, age, dietary habits, and body weight. Collected stool samples were kept in an icebox within 4 h of fecal reflex. The fecal samples were stored at -80°C in the lab until DNA extraction. The esophageal cancer patients and healthy subjects had no history of antibiotics, prebiotics, and probiotics intake 30 days before sample collection.

Bacterial DNA extraction by using fecal samples

QIAGEN (Germany, Hilden) stool kit was used for DNA extraction after thawing of stool sample with the initial bead-beating step (5000 rpm about 30 s). The bacterial DNA concentration was assessed by using Nano Photometer (Germany IMPLEN).

PCR augmentation for DGGE

PCR-DGGE was used to investigate the intestinal bacterial composition fingerprinting. For this purpose, the V3 region of the 16S rRNA gene of extracted DNA was amplified by using universal linkage primers (Suppl. Table 2). 50 μl PCR reaction mixture contained 200 mM of deoxynucleotide triphosphate (dNTP), 20 pmol of two primers, MgCl_2 2.5 mM, $10 \times$ buffer 2.5 mM, Taq 2 U of (Promega) DNA polymerase, and 2 μl (approximately 120 ng) stool bacterial DNA. PCR Amplification was carried out in thermocycler (USA ABI2720) through touchdown PCR programming. Agarose gel was used for the PCR product's electrophoresis and dipped in Ethidium bromide solution to visualize under UV light.

Denature gradient gel electrophoresis

Universal Mutation Detection System (Bio-Rad, USA) DCodeTM was used for the denaturing gradient gel electrophoresis experiment. Amplified PCR product of total bacteria of each stool sample was loaded in (w/v) 8% (37.5 : 1 acrylamide-bis,) DGGE polyacrylamide gel inside $1 \times$ TAE buffer tank, containing linear denaturing grade 30~65 %. The DGGE gel was electrophoresed at 90 V for 14 h at a constant temperature of 60°C . The bacterial diversity of each sample was calculated by using the relative band intensity of DGGE profiles and total numbers of bands. Syngene (USA) software was used to calculate the similarity index through Dice's similarity coefficient. Unweighted pair UPGMA (arithmetic averages) was used to establish a dendrogram.

Statistical analysis of DGGE band pattern

Bacterial diversity in DGGE profiles was assessed through the Shannon–Weaver index of diversity (H^1). Similarity index and DGGE profile's cluster analysis were done through the UPGMA method (band-based Dice similarity coefficient). Algorithm cluster analysis with (UPGMA) and arithmetic averages were used in the unweighted group pair method to estimate dendrograms.

Shannon–Weaver diversity index (H^1) was calculated as,

$$\text{Shannon–Weaver diversity index } (H^1) = - \sum_{i=1}^S (P_i)(\ln P_i)$$

Gel bands excision and sequencing

A sterilized scalpel blade was used to cut the prominent bands of the DGGE gel profile. The excised gel bands were collected in a 1.5 ml tube; approximately 50 ml sterilized water was added and placed for 30 min at 37°C . After centrifugation, the V3 region 16S rRNA gene was amplified by adding 8 μl DNA water (as a template) by using the same primers but without

GC-clamps. The PCR re-amplified product was evaluated by using ABI 3500xL for sequencing. Sequences were analyzed by applying BLAST database software to identify the genus and species.

qPCR analysis

Bio-Rad (USA) CFX96 was used to perform the qPCR quantification. Total 20 μl PCR reaction mixture contains 10 μl of $2 \times$ SYBR Green (TOYOBO, Japan), 1 μl of both primers (Suppl. Table 2), 2 μl fecal DNA, and 6 μl H_2O . *Lactobacillus* (NWS1), *Bifidobacterium* (CICC 6186), *Clostridium leptum* (YIT 6169), *Escherichia coli* (NWS2), and *Bacteroides vulgatus* (CICC 22938) were used as standard strains. Data Statistics were, the approximate average logarithm of fecal bacterial DNA genetic amplicon, copy numbers present in 1 g fecal mass.

Illumina based HiSeq 2500 High-throughput sequencing

Illumina-based high-throughput was applied to 20 randomly selected fecal samples for metagenomic analysis (10 patients' samples and 10 samples as control from healthy volunteers). According to the previous methodology, the 16S rRNA gene with V3+V4 location was amplified by using a primer: F-515-(GTGCCAGCMGCCGCGGTAA), R-806-(GGACTACH-VGGGTWTCTAAT) to manufacture the amplicon libraries. The paired-end High-throughput sequencing was performed on Illumina HiSeq 2500 platform using the manufacturer's standard procedure. Software packages FLASH and QIIME were used to screen and assembled the raw data. The UCLUST was computed to cluster OTUs sequences (Operational Taxonomic Units) with a 97% threshold. Also, a Classifier (RDP) was used to allot taxa level OTU. QIIME was used to study the diversities like Chao1, Good's coverage Simpson, Shannon, and ACE. The OTU cluster was manufactured by using the QIIME pipeline, which was then imported in MEGAN-4 and matched with the taxonomic database of NCBI, and fluctuations of the intestinal bacterial community were calculated.

Statistics analysis

Statistical analysis was performed in Microsoft Excel 2010 and statistic software GraphPad prism 7. A nonparametric unpaired *t*-test was computed, and significance value was measured as ($P < 0.05$).

Results

DGGE profiles analysis

Analytical and experimental process of denaturing gradient gel electrophoresis was performed with amplified PCR product by using the universal primers of the V3 region of the 16S rRNA gene in both esophageal cancer patients and healthy control groups. In Fig. 2A, (D1–D8) shows the esophageal cancer patients samples while (C1–C5) represents the healthy controls. Similarly, in Fig. 2C, (D9–D15) shows esophageal cancer patients' samples and (C6–C10) to healthy controls. A total of 194 bands were detected by using Syngene software (Van Der Gucht et al., 2001) in 15 tracks of esophageal cancer patients with a band average (12.12 ± 3.37). A sum of 71 bands was identified in 10 tracks of healthy volunteers group with an average of (7.73 ± 1.60), showed there was a significant band difference ($P < 0.0007$) between esophageal cancer and healthy control. The increased band numbers indicate the bacterial overgrowth in the esophageal cancer patients group. To inves-

tigate the intestinal microbial diversity between esophageal cancer patients and healthy control, the Shannon Weaver diversity index (H^1) was calculated. Diversity results illustrated (3.06 ± 0.69 vs. 2.52 ± 0.26) in a significant ($P < 0.017$) disparity between diseased and control groups also indicates a significant bacterial overgrowth in esophageal cancer patients as compared to healthy controls. Dice similarity coefficient and UPGMA dendrogram of the entire DGGE profiles were evaluated using Syngene software shown in Fig. 2B and Fig. 2D. Dice similarity coefficient based on band intensity of DGGE profile of esophageal cancer patients and control group, the mean similarity index comparison between two groups was (0.340 ± 0.164) and (0.271 ± 0.147), respectively (Suppl. Table 3). The mean similarity index between the two groups was (0.174 ± 0.155), indicating the lesser value in inter-group than intra-group, signifying that esophageal cancer patients' gut microbial composition was different from healthy individuals.

Dominant band analysis with sequencing results

A total of 23 gel bands were excised from two DGGE gel profiles. From Fig. 2A, 13 prominent bands were excised from the DGGE profile for quantity analysis. Bands belonging to different tracks but at a similar position, D3b and C3a, were cut and sequenced to confirm the resolution capacity of DGGE. It

was identified as *Prevotella copri* with 96% similarity. Similarly, from Fig. 2C, 10 bands were cut. Also, to check the resolution ability of DGGE gel, bands D13a and D14a were cut and sequenced and identified *Klebsiella pneumonia* with 93% similarity. Taxonomic reorganization of other bands is shown in Suppl. Table 4. BLAST software was used to analyze the sequence. Sequencing results demonstrated that the most prevalent phyla were Firmicutes, Proteobacteria, and Bacteroidetes. The two DGGE gel profiles' results indicated the prevalence of opportunistic bacteria (*Escherichia coli*, *Bacteroides vulgatus*, *Bacteroides fluxus*, *Enterobacter sacchari*, *Shigella flexneri*, *Proteus mirabilis*, *Bacteroides uniformis*, *Bacteroides salyersiae*, *Bacteroides plebeius*, *Bacteroides oleiciplenus*, *Klebsiella pneumonia*, *Bacillus* sp., *Pseudomonas cremoricolorata*, *Enterobacter aerogenes*, *Megasphaera* sp., *Bacteroides dorei*) in esophageal cancer patients.

qPCR quantification

qPCR showed the copy numbers of *Clostridium leptum*, *Bacteroides vulgatus*, *Lactobacillus* and *Bifidobacterium* genera. Results demonstrated that copy number of *Bifidobacterium* were decreased (6.91 ± 1.08 vs. 8.07 ± 1.04) significantly ($P < 0.0068$) in the esophageal cancer group as compared to healthy subjects, also *Lactobacillus* genus (7.12 ± 1.04 vs. 7.98 ± 0.89) was decreased significantly ($P < 0.0221$) in patients group. Copy

Fig. 2. (A) profiling of DGGE built between esophageal cancer (D1–D8) and controls counterparts (C1–C5). (B) Cluster estimation analysis between esophageal cancer (D1–D8) and healthy volunteers (C1–C5) groups by UPGMA application. (C) Profiling DGGE created between esophageal cancer (D9–D15) and healthy volunteers (C6–C10). (D) Cluster analysis between esophageal cancer (D9–D15) and healthy volunteers (C6–C10) groups, through UPGMA. “a” and “b” depicted in Fig. (2A) and (2C) linked with dominant bands of patients and controls. D and C represent esophageal cancer and healthy control groups, respectively.

numbers of *Bacteroides vulgatus* (7.93 ± 1.02 vs. 6.92 ± 1.03) were increased significantly ($P < 0.0119$), also replica count of *Escherichia coli* (4.59 ± 0.78 vs. 3.90 ± 0.85) were significantly higher ($P < 0.0241$) in study group as compared to controls, while those of *Clostridium leptum* (4.39 ± 1.18 vs. 4.12 ± 1.22) were increased non-significantly ($P < 0.2928$), Suppl. Table 5.

High-throughput sequencing analysis of gene sequence

The total sequence reads were assessed about 1,883,183 at (V3 + V4 location) 16S rRNA gene of 10 esophageal cancer patients and 10 from healthy volunteers. 1,577,154 High-throughput sequencing amplicons were selected after quality assurance (control 785,941, diseased 791,213, an average/sample 78857.7) were used for further investigation. The average Taxon in all samples was 74010.35. Unique tag in the control and study group were observed (1045.7, 1324.1), respectively, with an average unique tag (1184.9) in all esophageal cancer samples and healthy volunteers. Total 5541 OUT was allocated in the current study (disease 2946 and control 2595, average/sample 277.05). A total of 23698 unique tags demonstrated the whole intestinal bacterial community in this study. The average length of the High-throughput sequence was found about 418 (bp) after primer deletion.

Intestinal bacterial confirmation and diversification analysis

The bacterial diversity richness was calculated at a 97% similarity level. Alpha diversity variation was estimated by Shannon, Evenness ACE, Simpson, Observed species and Chao1 which were found significantly higher in esophageal cancer patients ($P < 0.0226$, $P < 0.0237$, $P < 0.0348$, $P < 0.0222$ and

$P < 0.0170$, respectively) as compared to the healthy volunteers. Conversely, Good's coverage was non-significantly higher in the disease group ($P < 0.0982$). The bacterial diversity estimation in the two groups is present in Suppl. Table 6. Moreover, alpha diversity exhibits a significant bullish trend in esophageal cancer patients compared to healthy individuals, clearly reflecting intestinal bacterial overgrowth in the diseased group. Each group of bacterial DNA samples was distributed into two distant clusters (on unweighted UniFrac tree metrics) shown in Fig. 3, corresponding to the PCR-DGGE pattern.

Phyla level taxa composition

The abundance of phylum, family, genus, and species is presented with a percentage (0.5–1%) in the current study. A total of 13 phyla were observed in High-throughput results. The significantly increased phylum was Bacteroidetes, while non-significantly decreased phyla were Firmicutes and Proteobacteria in the experimental group compared to healthy volunteers Fig. 4. Top 10 phyla statistics with percentage is shown in Suppl. Table 7, indicates the obvious dissimilarity between control and study group phyla.

Family level intestinal bacteria abundance

85 different families were found in High-throughput results; significantly increased families were Enterobacteriaceae, Bacteroidaceae, in the study group with a comparison of healthy volunteers. Whereas, the significantly lowered richness of Prevotellaceae and Veillonellaceae in the study group were observed in the comparison of control, depicted in Fig. 5. The 10 most families' statistics with percentages shown in Suppl. Table 7 display differences between disease and control groups.

Fig. 3. High-throughput sequencing diversity analysis among esophageal cancer samples and healthy counterparts (unweight UniFrac distances based on UPGMA). D and C symbolize esophageal cancer and healthy control groups, respectively.

Fig. 4. Intestinal bacterial composition at phylum levels from High-throughput sequencing outcomes. The relative abundance of prevalent phyla in esophageal cancer and controls. D and C depict esophageal cancer and healthy control groups, respectively.

Fig. 5. Family levels High-throughput results of intestinal bacterial conformations. Dominant families' relative abundance of esophageal cancer and normal controls. D and C show esophageal cancer and control group, respectively.

Genus level intestinal bacterial distribution

189 genera were observed in High-throughput sequencing. Significantly raised levels of genera were *Bacteroides*, and *Escherichia-Shigella* in the experimental group, contrary to control. Nevertheless, significantly decreased genera in results were *Prevotella_9* and *Dialister*, as shown in Fig. 6, Supp. Table 7. Esophageal cancer affects Phylum Bacteroidetes, family Veillonellaceae, Prevotellaceae and Enterobacteriaceae, genera

Bacteroides, *Prevotella_9*, *Escherichia-Shigella*, and *Dialister*. It also affects the other intestinal bacteria that may change the health status of an individual. Species results exhibited a notable difference between esophageal cancer patients and controls. Conversely, significant enrichment of *Bacteroides vulgatus* and *Escherichia coli* in esophageal cancer patients is depicted in Suppl. Table 8.

Fig. 6. Genera level intestinal bacterial compositions by High-throughput sequencing outcomes. The relative abundance of the most prevalent genera in esophageal cancer and healthy controls. D and C denote esophageal cancer and healthy control groups, respectively.

Discussion

The gut microbiome plays a vital role in the normal physiological function of the human body. There is growing evidence that gut microbial composition undergoes alteration in disease conditions, such as Crohn's disease, malnutrition, inflammatory bowel disease, colitis, obesity, and type II diabetes. On the other side, Esophageal cancer is a fast-growing tumor and it is estimated that it is the 6th leading cause of death in humans (Mao, 2016). Recently, the microbiome emerged as a critical player and driver of various types of cancer, including intestine, breast, and lung cancer.

It was hypothesized that gut flora influences the (ROS) reactive oxygen species production through immune cells. So, ROS increases the DNA damaging chance produced via oxalipatin. This process would block the transcription and DNA replication, which results in cell death (Iida et al., 2013). In the present study, we observed a clear relationship between the gut microbiome and esophagus cancer. The results were authenticated by DGGE, qPCR, and High-throughput sequencing analysis. In α -diversity, Shannon, Simpson, observed species Chao1, ACE, Evenness showed a significant elevation of the unique microbial population in esophageal cancer patients (Ishaq et al., 2017a).

The DGGE gut microbial similarity index statistics in esophageal cancer patient was found more abundant in intra-groups, which indicate the overgrowth of bacteria. The similarity index assessment comparison is significantly lower in the inter-group against intra-group, agreeing with previously reported results (Wu et al., 2010). Thus, the above diversity findings showed significant gut microbial dysbiosis between esophageal cancer patients and healthy volunteers. Moreover, the quantitative statistical data also showed a considerable alteration between esophageal cancer patients and control. A gut

microbiome meta-analysis has been linked with irritable bowel syndrome (IBD) and obesity. It revealed that the percentage of Firmicutes and Bacteroidetes is not stable from lean to obese intestinal bacteria (Walters et al., 2014). A higher abundance of Bacteroidetes has also been linked with diseases like type II and type I diabetes and irritable bowel syndrome (Pittayanon et al., 2019). Usually, a healthy human esophagus contains a distinct microbial flora than the oral microbiome. Also, the normal esophagus possesses the Phylum Firmicutes as a significant part of the microbiota. Nevertheless, the prevalence of other phyla like Proteobacteria, Bacteroides, Actinobacteria, Fusobacteria has also been reported in previous literature (Baba et al., 2017). However, our finding showed a significant decrease and increased level of Firmicutes, Proteobacteria, and Bacteroides in gut microbiota of esophageal cancer patients compared to the healthy counterparts.

If we see the microbial families, Veillonellaceae and Prevotellaceae were significantly lesser in esophageal cancer patients than healthy volunteers aligning with current research work (Chen et al., 2016; Zhang et al., 2017). Veillonellaceae is a beneficial microbe that is closely associated with *Clostridium*. It helps in the production of T-regulatory immune cells (Atarashi et al., 2011). Gut microbiota plays a vital role in the immune function of the body. So, it influences the successful PDL1 (antibody) immunotherapy of cancer care patients (Garrett, 2015; Iida et al., 2013). However, a significantly higher abundance of Bacteroidaceae and Enterobacteriaceae were observed in esophageal cancer patients compared to healthy volunteers, which is aligned with preceding research of type-II diabetes individuals and intestinal flora (Wang et al., 2017).

Conversely, genera *Dialister* and *Prevotella_9* were decreased significantly in the study group as compared to control. Lowered level *Prevotella* is linked with types-1 diabetes and autism and gut microbiome (Kang et al., 2013). Significantly increased genera were *Bacteroides* and *Escherichia-Shi-*

gella, in the patient's group compared to control, agreeing with reported research of autism disorders and intestinal bacteria (Strati et al., 2017).

The current study indicates the significant reduction of *Prevotella* genera in the study group compared to control; *Prevotella* has a beneficial role in an individual's metabolism (Kovatcheva-Datchary et al., 2015). However, *Prevotella* is a useful commensal bacteria related to plant-based food, also linked with inflammation (Dillon et al., 2015). The results showed significantly increased values of *Escherichia-Shigella* in esophageal cancer patients as compared to healthy counterparts. However, *Escherichia-Shigella* always produces Shiga-toxin, which may cause colitis, septicemia, thrombocytopenia, GIT inflammations, urinary tract infection (Amani et al., 2015). Moreover, the raised level of *Escherichia coli* can also cause food-borne diseases and (UTIs) urinary tract infections (Mulvey et al., 2000). It has been documented that there is a high prevalence of *Escherichia coli* in esophageal carcinoma and Barrett's esophagus. Current research also showed a significantly raised level of *Escherichia coli* in the gut of esophageal cancer (Zaidi et al., 2016). Moreover, it has been reported TLR 1-3, 6, 7, and 9 is notably upregulated in cancer like esophageal adenocarcinoma compared to the normal healthy epithelium. It indicates the possible linkage between *Escherichia coli* and TLR signaling pathways. The microbes can mediate early molecular modifications in an experimental mouse model of esophageal adenocarcinoma carcinogenesis (Zaidi et al., 2016). However, a significantly raised abundance of *Bacteroides vulgatus* species was observed in the patient's group, which is in accordance with previous literature on viral diarrhea and gut microbiome (Ma et al., 2011). It might be the possible causative intestinal bacteria in the esophageal cancer patient. However, there is an elevated threat of colon carcinoma that is interlinked with the high existence of *Bacteroides vulgatus* (Guarner and Malagelada, 2003).

The current study findings elaborated the significant alteration of phylum, family, genus, and species between esophageal cancer patients and control (Manichanh et al., 2008). The results indicate esophageal cancer may alter the intestinal physiology, leading to a change in the intestinal bacterial configuration. Furthermore, these variations in the intestinal bacterial composition may cause worsening of illness (Qin et al., 2010). Esophageal cancer patients' clinical signs are indigestion, vomiting, difficulty in swallowing, choking, etc. It would also be hypothesized that esophageal cancer might alter the intestinal bacterial confirmation, particularly Phylum Bacteroidetes, family Bacteroidaceae Prevotellaceae Enterobacteriaceae Veillonellaceae, genera *Dialister*, *Prevotella_9*, *Escherichia-Shigella*, species *Bacteroides vulgatus*, and *Escherichia coli*, also influence the whole gut bacteria. As the microbial flora influences the progression and development of esophageal carcinoma (Baba et al., 2017). Consequently, the microbiota has the potential capacity to alter the efficacy and toxicity of chemotherapy (Carmody and Turnbaugh, 2014). Current research is pretty motivating because esophageal cancer and intestinal bacteria have no direct linkage. These alterations in gut bacteria may change the host health even though illness progress is not related to the intestine (Nam et al., 2013).

Also, qPCR was performed to investigate the quantitative modification of intestinal bacteria. Results demonstrated a significant decrease of Bifidobacterium and Lactobacillus genera in esophageal cancer patients associated with preceding work (Zhou et al., 2014). Additionally, a substantial increase of *Bacteroides vulgatus*, and *E. coli*, while the insignificant rise of *Clostridium leptum* in the study group compared to control, is

analogous to previous studies (Guarner and Malagelada, 2003; Ma et al., 2011; Wu et al., 2010).

Conclusions

In conclusion, the current study revealed significant intestinal microbiome dysbiosis between esophageal cancer patients and healthy volunteers. More specifically, there is a considerable disparity of intestinal bacterial taxa composition with the comparison of controls. The degree of certain intestinal bacteria was either showed significant increased or lowered abundance in esophageal cancer patients. The estimation of intestinal bacterial diversity shows a significantly higher level in intestinal flora of esophageal cancer than healthy volunteers, which indicates the overgrowth of gut bacteria in esophageal cancer patients. Thus, further study is required to understand the fundamental processes and mechanism of gut bacterial dysbiosis in esophageal cancer patients.

Author contribution

Hafiz Muhammad Ishaq, Jiru Xu: *Study concept and design*. Hafiz Muhammad Ishaq: *Data acquisition*. Hafiz Muhammad Ishaq, Imran Shair Mohammad: *Manuscript preparation*. Kiran Sher Muhammad, Huan Li, Rao Zahid Abbas, Zia ud Din Sindhu, Shakir Ullah, Yang Fan, Abbas Sadiq, Muhammad Asif Raza, Riaz Hussain, Hafiz Muhammad Arshad, Iahtasham Khan, Muhammad Umair Waqas, Aziz Ul-Rahman, Riffat Yas-in, Atif Rehman, Rana Waseem Akhtar: *Manuscript editing*. All authors: *Manuscript review*.

Conflict of interests

All the authors declare no conflict of interests.

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Supplementary material

Table 1. The type and grade of esophageal cancer patients recruited

Patient	Age	Sex	Type of carcinoma	Cancer grade
1	55	Male	Squamous cell carcinoma	Grade 2
2	60	Male	Squamous cell carcinoma	Grade 3
3	45	Male	Adenocarcinoma	Grade 3
4	53	Male	Squamous cell carcinoma	Grade 2
5	55	Male	Adenocarcinoma	Grade 2
6	51	Male	Squamous cell carcinoma	Grade 2
7	56	Male	Squamous cell carcinoma	Grade 2
8	55	Male	Squamous cell carcinoma	Grade 2
9	49	Male	Squamous cell carcinoma	Grade 3
10	52	Male	Adenocarcinoma	Grade 2
11	60	Female	Squamous cell carcinoma	Grade 2
12	52	Female	Squamous cell carcinoma	Grade 2
13	57	Female	Squamous cell carcinoma	Grade 3
14	46	Female	Adenocarcinoma	Grade 2
15	53	Female	Squamous cell carcinoma	Grade 2

Table 2. Linkage Primers deployed in PCR-DGGE and qPCR

Target bacteria	Primer Sequence (5'–3')	
PCR-DGGE Primer		
341-F	CCTACGGGAGGCAGCAG	
534-R	ATT ACCGCGGCTGCTGG	
341FG	CGCCCGCCGCGCGCGGCGCGGGGCGGGGGCAGGGGGGCTACGGGAGGCAGCAG	
Real-Time PCR Primer		
<i>Bifidobacterium</i> (550 bp)	Bifd F	CTC CTGGAACGGGTGG
	Bifi-R	GGTGTCTCTCCCGATATCTACA
<i>Lactobacillus</i> (250 bp)	Lact F	CTCAAACTAAACAAAGTTTC
	Lact R	CTCAAACT AAACAAAGTTTC
<i>Bacteroides vulgatus</i> (287 bp)	BV-F	GCATCATGAGTCCGCATGTTC
	BV-R	TCCATACCCGACTTTATTCCTT
<i>Escherichia coli</i> (287 bp)	E. coli-F	CATTGACGTTACCGCAGAAGAAGC
	E. coli-R	CTCTACGAGACTCAAGCTTGC
<i>Clostridium leptum</i> (239 bp)	C. lep-F	GCACAAGCAGTGGAGT
	C. lep-R	CTTCCTCCGTTTTGTCAA

Table 3. Gut microbial diversity and similarity of esophageal cancer patients and healthy control

Groups	Diversity		Similarity	
	Number of bands ^a	Shannon index ^b	Intra-similarity ^c	Inter-similarity ^d
Patients group	12.12 ± 3.37	3.06 ± 0.69	0.340 ± 0.164	0.174 ± 0.155
Healthy control	7.73 ± 1.60	2.52 ± 0.26	0.271 ± 0.147	
P-value	0.0007	0.017	/	/

Significantly different through, unpaired *t* test, with ($P < 0.05$).

^a DGGE bands number produced by each sample; ^b Shannon–Weaver diversity index (H') was evaluated in each sample by using (relative) intensities of all DGGE bands; ^c Comparing of DGGE band profiles with Dice similarity coefficients within the individual of a given group; ^d Comparing of DGGE band profiles by using Dice similarity coefficients between members of esophageal cancer patients and healthy group.

Table 4. Sequencing of excised PCR-DGGE bands and identification by using the BLAST database

Selected excised bands	Bacteria with highest % homology	Sequence accession number	Bacterial phyla	Gene bank number
D1a	<i>Escherichia coli</i> (98)	O104:H4	Proteobacteria	NC_018658.1
D2a	<i>Bacteroides fluxus</i> (89)	YIT 12057	Bacteroidetes	NZ_GL882626.1
D3a	<i>Enterobacter sacchari</i> (96)	SP1	Firmicutes	NZ_CP007215.2
D3b	<i>Prevotella copri</i> (96)	DSM 18205	Bacteroidetes	NZ_GG703856.1
D4a	<i>Shigella flexneri</i> (98)	2a str. 301	Proteobacteria	NC_004337.2
D4b	<i>Bacillus cecembensis</i> (98)	DSM 21993	Firmicutes	NZ_LMBZ01000203.1
D5a	<i>Proteus mirabilis</i> (94)	HI4320	Firmicutes	NC_010554.1
D6a	<i>Bacteroides vulgatus</i> (97)	ATCC 8482	Bacteroidetes	NC_009614.1
D7a	<i>Bacteroides uniformis</i> (95)	CL03T00C23	Bacteroidetes	NZ_JH724260.1
D8a	<i>Bacteroides salyersiae</i> (94)	DSM 18765	Bacteroidetes	NZ_KB905470.1
C1a	<i>Bacteroides stercoris</i> (94)	ATCC 43183	Bacteroidetes	NZ_DS499676.1
C3a	<i>Prevotella copri</i> (96)	DSM 18205	Bacteroidetes	NZ_GG703856.1
C4a	<i>Bacteroides coprocola</i> (97)	DSM 17136	Bacteroidetes	NZ_DS981495.1
D9a	<i>Bacteroides plebeius</i> (91)	DSM 17135	Bacteroidetes	NZ_DS990123.1
D9b	<i>Bacteroides oleiciplenus</i> (91)	YIT 12058	Bacteroidetes	NZ_JH992946.1
D13a	<i>Klebsiella pneumonia</i> (93)	HS11286	Firmicutes	NC_016845.1
D14a	<i>Klebsiella pneumonia</i> (93)	HS11286	Firmicutes	NC_016845.1
D15a	<i>Bacillus</i> sp. (99)	FJAT-25496	Firmicutes	NZ_LMBY01000086.1
D15b	<i>Pseudomonas cremoricolorata</i> (98)	ND07	Proteobacteria	NZ_CP009455.1
D15C	<i>Enterobacter aerogenes</i> (92)	KCTC 2190	Proteobacteria	NC_015663.1
D15b	<i>Megasphaera</i> sp. (94)	DJF_B143	Firmicutes	NZ_LODR01000015.1
C6a	<i>Bacteroides dorei</i> (92)	HS2L2B045b	Firmicutes	CP009057.1
C10a	<i>Bacteroides caecimuris</i> (92)	I48	Bacteroidetes	NZ_CP015401.1

Table 5. Different bacterial quantification outcomes by qPCR

Bacteria	Control	Patients	P-value
<i>Bifidobacterium</i> (10^5)	8.07 ± 1.04	6.91 ± 1.08	0.0068
<i>Bacteroides vulgatus</i> (10^7)	6.92 ± 1.03	7.93 ± 1.02	0.0119
<i>Lactobacillus</i> (10^4)	7.98 ± 0.89	7.12 ± 1.04	0.0221
<i>Escherichia coli</i>	3.90 ± 0.85	4.59 ± 0.78	0.0241
<i>Clostridium leptum</i> (10^5)	4.12 ± 1.22	4.39 ± 1.18	0.2928

Data presented as the average estimate of logarithms of fecal PCR target genetic amplicon, copy numbers present in 1 g of feces.

Table 6. High-throughput intestinal bacterial diversity index based on the 97% similarity analysis

Group	Observed species	OTUs	Shannon	Simpson	Chao1	ACE	Good' coverage	Evenness
Patients	278.6	288.1	5.05	0.943	289.23	295.59	0.9997	0.387
Control	253.8	259.5	4.35	0.909	257.00	268.03	0.9994	0.334
P-value	0.0174	0.0414	0.0226	0.0222	0.0170	0.0348	0.0982	0.0237

The mean values of each group shown in the table, unpaired *t*-test *t* were used to signify the results, where ($P < 0.05$). The evenness based on Shannon, calculated by using formula $E = H/\ln(S)$; where *H* denotes Shannon index of diversity and *S* represents whole number sequences in a particular group.

Table 7. Esophageal cancer intestinal bacterial phylotypes obtained from High-throughput sequencing

Taxa	Mean D	Mean C	P-value	Q-value	% D	% C
<i>Phylum</i>						
Firmicutes	0.33616	0.44690	0.15313	0.74408	33.62	44.69
Bacteroidetes	0.58414	0.34977	0.03143	0.63178	58.41	34.98
Proteobacteria	0.07261	0.15099	0.08494	0.63315	7.26	15.10
Verrucomicrobia	0.00108	0.01061	0.67300	0.96243	0.11	1.06
Tenericutes	0.00165	0.01003	0.32144	0.68118	0.17	1.00
Cyanobacteria	0.00020	0.01007	0.33450	0.76168	0.02	1.01
Actinobacteria	0.00319	0.01047	0.15139	0.76178	0.32	1.05
Fusobacteria	0.00069	0.00115	0.53111	0.71220	0.07	0.11
Acidobacteria	0.00011	0.00881	0.10569	0.74038	0.01	0.88
Synergistetes	0.00001	0.00104	0.27426	0.69438	0.00	0.10
Others					0.02	0.01
<i>Family</i>						
Bacteroidaceae	0.35432	0.17513	0.03211	0.73533	35.43	17.51
Ruminococcaceae	0.14991	0.20079	0.22533	0.70320	14.99	20.08
Prevotellaceae	0.03579	0.19696	0.02641	0.64459	3.58	19.70
Lachnospiraceae	0.09669	0.09224	0.43025	0.75133	9.67	9.22
Enterobacteriaceae	0.06367	0.02065	0.04321	0.69323	6.37	2.07
Alcaligenaceae	0.05654	0.06651	0.42342	0.86213	5.65	6.65
Veillonellaceae	0.02649	0.06263	0.03751	0.61437	2.65	6.26
Porphyromonadaceae	0.06416	0.05181	0.71409	0.89288	6.42	5.18
Rikenellaceae	0.06186	0.05754	0.64235	0.95458	6.19	5.75
unidentified_Chloroplast	0.02112	0.01011	0.82317	0.84133	2.11	1.01
Others					6.95	6.56
<i>Genus</i>						
Bacteroides	0.30432	0.20513	0.04361	0.51558	30.43	20.51
Prevotella_9	0.03349	0.18523	0.02183	0.61072	3.35	18.52
Alistipes	0.06186	0.05754	0.71837	0.86348	6.19	5.75
Escherichia-Shigella	0.05052	0.00155	0.03540	0.63280	5.05	0.16
Ruminococcus_2	0.02725	0.03395	0.81108	0.88648	2.73	3.40
Dialister	0.00813	0.04356	0.03677	0.62039	0.81	4.36
Parasutterella	0.05810	0.02785	0.13753	0.61528	5.81	2.78
Faecalibacterium	0.04549	0.02653	0.11030	0.72153	4.55	2.65
Parabacteroides	0.03649	0.03189	0.51916	0.87121	3.65	3.19
[Eubacterium]_coprostanoligenes_group	0.02417	0.03251	0.57431	0.92243	2.42	3.25
Others					35.02	35.43

Significantly dissimilar $P < 0.05$, D and C represent esophageal cancer and control.

Table 8. Species levels intestinal bacterial richness by High-throughput outcomes

Taxa	Mean D	Mean C	P-value	Q-value
<i>Bacteroides vulgatus</i>	0.211467	0.032123	0.03461	0.62015
<i>Escherichia coli</i>	0.071835	0.011431	0.03262	0.64025
<i>Bacteroides uniformis</i>	0.065836	0.023431	0.06444	0.74025
<i>Bacteroides plebeius</i>	0.026473	0.015168	0.20426	0.86106
<i>Bacteroides stercoris</i>	0.033955	0.021475	0.0464	0.73016
<i>Bacteroides coprophilus</i>	0.015334	0.005112	0.0753	0.72186
<i>Bacteroides massiliensis</i>	0.012785	0.004763	0.4156	0.87008
<i>Parabacteroides distasonis</i>	0.031134	0.014976	0.31257	0.83664
<i>Haemophilus parainfluenzae</i>	0.015944	0.008915	0.32418	0.89188
<i>Sutterella wadsworthensis</i>	0.021251	0.001865	0.13455	0.70193
<i>Fritillaria taipaiensis</i>	0.022116	0.000211	0.13104	0.99178
<i>Lactobacillus casei</i>	0.001267	0.005583	0.03174	0.71172
<i>[Clostridium] leptum</i>	0.012471	0.007118	0.13366	0.71766
<i>Paraprevotella clara</i>	0.00056	0.004652	0.02796	0.28954
<i>Ruminococcus bicirculans</i>	0.004221	0.006382	0.56024	0.87372
<i>Bacteroides fragilis</i>	0.009088	0.003979	0.15782	0.67786
<i>salivarius subsp. thermophilus</i>	0.008903	0.000342	0.1118	0.5862
<i>Bifidobacterium longum</i>	0.000766	0.004095	0.16671	0.74025
<i>Bacteroides ovatus</i>	0.03718	0.01544	0.07581	0.52122
<i>Bifidobacterium kashiwanohense</i>	0.001315	0.005971	0.1049	0.4951
<i>Shigella flexneri</i>	0.013466	0.012497	0.57631	0.88965
<i>Bacteroides dorei</i>	0.003696	0.000327	0.27551	0.82114
<i>Paraprevotella clara</i>	0.00054	0.004562	0.02885	0.28754
<i>Phascolarctobacterium faecium</i>	0.007023	0.002458	0.16751	0.84117
<i>Dialister propionificiens</i>	6.58E-04	1.05E-04	0.01896	0.34236
<i>Megasphaera micronuciformis</i>	0.000212	7.18E-04	0.03894	0.43561
<i>Ruminococcus sp._5_1_39BFAA</i>	0.002698	0.001381	0.21856	0.82117
<i>Alistipes shahii</i>	0.01263	0.009748	0.4023	0.88107
<i>Alistipes indistinctus</i>	0.000718	0.002913	0.35365	0.85024

Significantly dissimilar $P < 0.05$.