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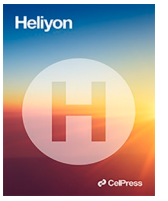

Research article

Impact of the gut microbiome on response and toxicity to chemotherapy in advanced esophageal cancer

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A R T I C L E I N F O

Keywords:
Gut microbiota
Esophageal squamous cell carcinoma
Chemotherapy
Efficacy
Toxicity

A B S T R A C T

Objective: To identify the gut bacteria associated with chemotherapeutic outcomes, t characterized the gut microbiota in patients with esophageal squamous cell carcinoma (ESCC) in this prospective study.

Design: Thirty-one patients with ESCC were enrolled. Chemotherapy was performed with paclitaxel and cisplatin (TP). Fecal samples were collected before and after treatment and analyzed using 16S rRNA sequencing.

Results: The species with differences in baseline abundance between partial response (PR) and non-PR groups was identified as *Bacteroides plebeius* ($P = 0.043$). The baseline abundance of *B. plebeius* was higher in the responder (R, PR + stable disease (SD)) group ($P = 0.045$) than in the non-responder (NR). The abundance of *B. ovatus* was identified as a predictor for distinguishing patients with PR from those without PR (sensitivity, 83.3 %; specificity, 69.6 %). The abundance of *B. plebeius* was positively associated with the response to PR + SD (R) in predicting responders in the receiver operating characteristic (ROC) curve analysis (area under the ROC curve = 0.865, $P = 0.041$). The abundance of *B. plebeius* and *B.uniform* was a predictor of grade (G) 3–4 chemotherapy toxicities. The sensitivity and specificity of the established multi-analyte microbial predictive model demonstrated a better predictive ability than a single parameter (*B. uniform* or *B. plebeius*).

Conclusion: The abundance of gut microbiota *B. plebeius* and *B. ovatus* are associated with the efficacy of TP chemotherapy in patients with ESCC. The abundance of *B. plebeius* and *B.uniform* may related to the toxicity of TP chemotherapy.

1. Introduction

The incidence and mortality rates of digestive tract tumors are high worldwide. The incidence of esophageal cancer (EC) in East Asia ranks first in the world, reaching 24.7/105, which is three times than that in Europe and North America [1]. EC ranks sixth in

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terms of incidence in China. Its mortality rate ranks among the top three digestive tract tumors. In 2020, 324,000 new cases of EC and 301,000 deaths were recorded in China [2], with a mortality rate of 92.9 %. Common histological types include squamous cell carcinoma (SCC), adenocarcinoma, and esophageal SCC (ESCC), which comprise approximately 90 % of cases in China. Radical surgery is not recommended for >60 % of patients with ESCC with locally advanced or distant metastatic disease at diagnosis. Currently, the main treatment approaches for advanced EC include chemotherapy, targeted therapy, and immune checkpoint inhibitor therapy. However, its efficacy remains limited. Chemotherapy is the basis of systemic therapeutic strategies.

Several studies have indicated that the occurrence and development of digestive tract tumors are associated with multiple factors such as genetic or epigenetic variations, immune status, dietary and environmental factors, and microbial infections [3]. Over the past decade, many studies have explored biomarkers associated with the therapeutic efficacy of digestive tract tumors [4]. However, owing to the high prevalence of ESCC in East Asia, large-scale global research is limited. Therefore, exploring new biomarkers for predicting the therapeutic efficacy of ESCC, particularly for predicting chemotherapy response, has become an important issue.

The human gut is a complex biological system, with approximately 3.8×10^{13} species of microorganisms, mainly composed of bacteria, archaea, fungi, protozoa, and viruses. These microorganisms participate in physiological activities such as maintaining normal physiological functions of the human body and regulating immunity and metabolism [5,6]. Increasing evidence has demonstrated that gut microorganisms are associated with tumors [7]. The gut microbiota plays a key role in the occurrence and development of gastrointestinal tumors. Some bacteria in the intestine may produce toxins that induce DNA damage and genetic instability in the digestive tract epithelial cells, thereby promoting colon carcinogenesis [8]. Lithocholic and deoxycholic acids produced by bacteria in the distal small intestine and colon can be activated by triggering the NFκB signal pathway in colon epithelial cells to contribute to carcinogenesis [9]. Some intestinal bacteria produce beneficial butyrate that inhibits the occurrence of colitis and tumors [10]. Dysbiosis of the intestinal microbiota can trigger a series of innate and adaptive immune responses, affecting T-cell differentiation, regulating the expression of interleukins 10 and 23, and activating STAT3 signaling pathways, leading to tumorigenesis [11].

Clinical studies have reported that *Ruminococcus* [12], *Faecalibacterium* [13], and *Bifidobacteria* [14] are significantly reduced in the fecal microbiota of patients with colorectal cancer compared to that of healthy people. By contrast, the abundance of *Bacteroides fragilis* [15] and *Escherichia coli* [8] is increased in patients with colorectal cancer. *E. coli* and *Fusobacterium nucleatum* were detected in ESCC tissues. The abundance of *Clostridium nucleatum* is negatively associated with the survival of patients with ESCC [16], indicating that some specific bacteria may influence the occurrence and development of upper digestive tract tumors.

Studies have showed that microbiome also affects the efficacy of anti-tumor therapy. The anti-tumor effect of cyclophosphamide is partially achieved by changing the gut microbiota and promoting the translocation of specific G^+ bacteria, which stimulate the production of pTh17 cell subsets and activate anti-tumor immunity [17]. The efficacy of oxaliplatin also related to the activation of myeloid cells by gut microbiota and the release of reactive oxygen species, which play genotoxic and tumor-suppressive roles [18]. A previous study has reported that mice with melanoma treated with programmed death-ligand 1 (PD-L1) antibody had a better tumor regression response in mice with a higher abundance of Bifidobacteria in the intestine, and the activity of cluster of differentiation 8 (CD8+T) cells in the body was significantly enhanced [19]. Studies have indicated that the abundances of *Ruminococcus* and *Collinsella* in the intestines of patients with melanoma positively correlate with the efficacy of immunotherapy [20,21]. In studies focusing on lung and renal cancers, the progression-free survival (PFS) and overall survival (OS) related to immunotherapy in patients receiving antibiotics have been significantly shortened. Moreover, antibiotics may reduce the efficacy of immunotherapy by causing dysbiosis [22]. Patients with cancer often receive chemotherapy that alters their gut microbiota [23]. XELOX chemotherapy, including oxaliplatin and capecitabine, alters the composition of the gut microbiota [24]. *Klebsiella pneumoniae* is commonly detected in patients with chemotherapy-induced diarrhea (25. In addition, *Enterobacteriaceae* are associated with the promotion of mucositis, a common chemotherapy toxicity [26]. Adverse reactions can lead to dose reduction during chemotherapy, which may affect the clinical outcomes. Thus, obtaining comprehensive knowledge of the association between the microbiome and chemotherapy toxicity is crucial in clinical practice.

To date, few studies have focused on the association between intestinal microbiota and tumors of the upper gastrointestinal tract, particularly ESCC, in Asian populations. Moreover, evidence supporting the application of biomarkers for predicting the efficacy and toxicity of chemotherapeutic drugs in patients with ESCC is insufficient. To identify specific bacteria associated with chemotherapeutic efficacy and drug toxicity, we characterized the gut microbiota of patients with ESCC receiving chemotherapy regimens in this prospective study.

2. Materials and methods

2.1. Participants and sample collection

Altogether, 31 patients with ESCC scheduled for chemotherapy were prospectively enrolled in the Department of Oncology at Peking Union Medical College Hospital (PUMCH) between 2018 and 2020. Patients newly diagnosed with ESCC, confirmed as locally advanced or metastastic disease according to the tumor–node–metastasis classification (American Joint Committee on Cancer 7.0) were considered eligible. Patients who lived in northern China and maintained an oral diet were eligible. The exclusion criteria included bowel obstruction, infection, inflammatory bowel disease, chronic disease, antibiotic/probiotic medication within 1 month before fecal sample collection, and long term medication history.

All the participants provided written informed consent before study participation. The study was conducted in accordance with the Declaration of Helsinki (revised in 2013) and was approved by the Ethics Committee of PUMCH.

The patients received chemotherapy every three weeks. Chemotherapy was administered with TP (paclitaxel 175 mg/m^2 on day 1;

cisplatin 25 mg/m^2 days 1–3). Efficacy was evaluated 6 weeks after chemotherapy initiation according to the Response Evaluation Criteria for Solid Tumors (RECIST 1.1). Clinical data were collected from medical records. Fecal samples were collected from the patients within 3 days before chemotherapy (named "baseline"), and the second sampling was at 6 weeks after treatment (named "after treatment"). Approximately 50–100 mg of fecal samples were self-collected in an MGIEasy collecting tube and stored in −80 °C until further processing.

2.2. Extraction of fecal DNA and 16S rRNA sequencing

Genomic DNA was extracted using the NucleoSpin Soil DNA Kit (Macherey-Nagel Vertrieb Gmbh & Co. Kg., Germany). Polymerase chain reaction (PCR) amplification was performed by primers specific for the V4 variable region (515F: 5′GTGCCAGCMGCCGCGGTAA3'; 806R: 5′GGACTACHVGGGTWTCTAAT3′) [27] of the 16S rRNA gene. Purified PCR products (Qiagen) were used to construct a library. Our PCR conditions used and sequencing approach is analogous to the approach described elsewhere [27]. Illumina HiSeq2500 gene sequencing analysis system and PE250 sequencing strategy were applied to the qualified libraries.

2.3. Microbiota data analysis

The raw sequence data were filtered to remove low-quality reads. Forward and reverse pairwise reads were incorporated into tags using the FLASH software ((v1.2.11, http://ccb.jhu.edu/software/FLASH/index.shtml/FLASH-1.2.11.tar.gz) [28]. The tags were clustered into operational taxonomic units (OTUs) using the USEARCH software (v7.0.1090; http://www. drive5.com/usearch) at 97 % similarity level. The GreenGene Database (V201305; http://greengenes.secondgenome.com) [29] was used as a reference for species identification to compare the selected OTUs.

We applied alpha diversity statistics to compare the microbiota communities and demonstrate their abundance and homogeneity (Shannon's index, R software package v. 3.1.1). Analysis of similarity (ANOSIM) was applied to compare microbial diversity among the groups. Bar plots, partial least squares discriminant analysis, and heatmaps demonstrate the differences in microbial abundance between groups with different chemotherapy efficacies and survival.

2.4. Statistical analysis

We used the R software package (v. 3.1.1) and SPSS statistics 22.0 to perform statistical analysis. The Wilcoxon rank-sum test was used to calculate microbial differences between the two groups. Significance was set at $P < 0.05$. Kaplan–Meier estimates and log-rank tests were used for survival analysis. Receiver operating characteristic (ROC) curves were used to identify the ability of specific bacteria to distinguish between patients with different chemotherapeutic efficacy and survival. The cutoff values were estimated at

Table 1
Characteristics of ESCC patients.

Features	Esophageal cancer (n = 31)
Age	
Middle(45–59)	7
Elderly(>59)	24
Gender (Male/Female)	27/4
Differentiation	
Poorly	5
Middle-High	26
Staging of patients	
Locally advanced	22
Distant metastases	9
Metastasis sites	
0	22
1	4
≥2	5
ECOG PS	
0-1	19
2-4	12
Adverse reaction of therapy	
Grade 1-2	22
Grade 3-4	9
Response	
PR	7
SD	18
PD	6

ESCC, esophageal squamous cell carcinoma; ECOG, Eastern Cooperative Oncology Group; PS, Performance Status; PR, partial response; SD, stable disease; PD, disease progression.

various sensitivities and specificities and were determined using the maximized Youden's index (Sensitivity + Specificity − 1).

3. Results

3.1. Clinical characteristics of the patients

Altogether, 31 patients with ESCC aged 49–72 years and comprising 27 men and 4 women were included in this study. Among them, 22 cases were confirmed as having locally advanced disease, and 9 presented with distant metastases. Nine patients experienced grade 3–4 adverse reactions after TP chemotherapy. Grade 3–4 (G3–4) myelosuppression was observed in 7 patients, while G3

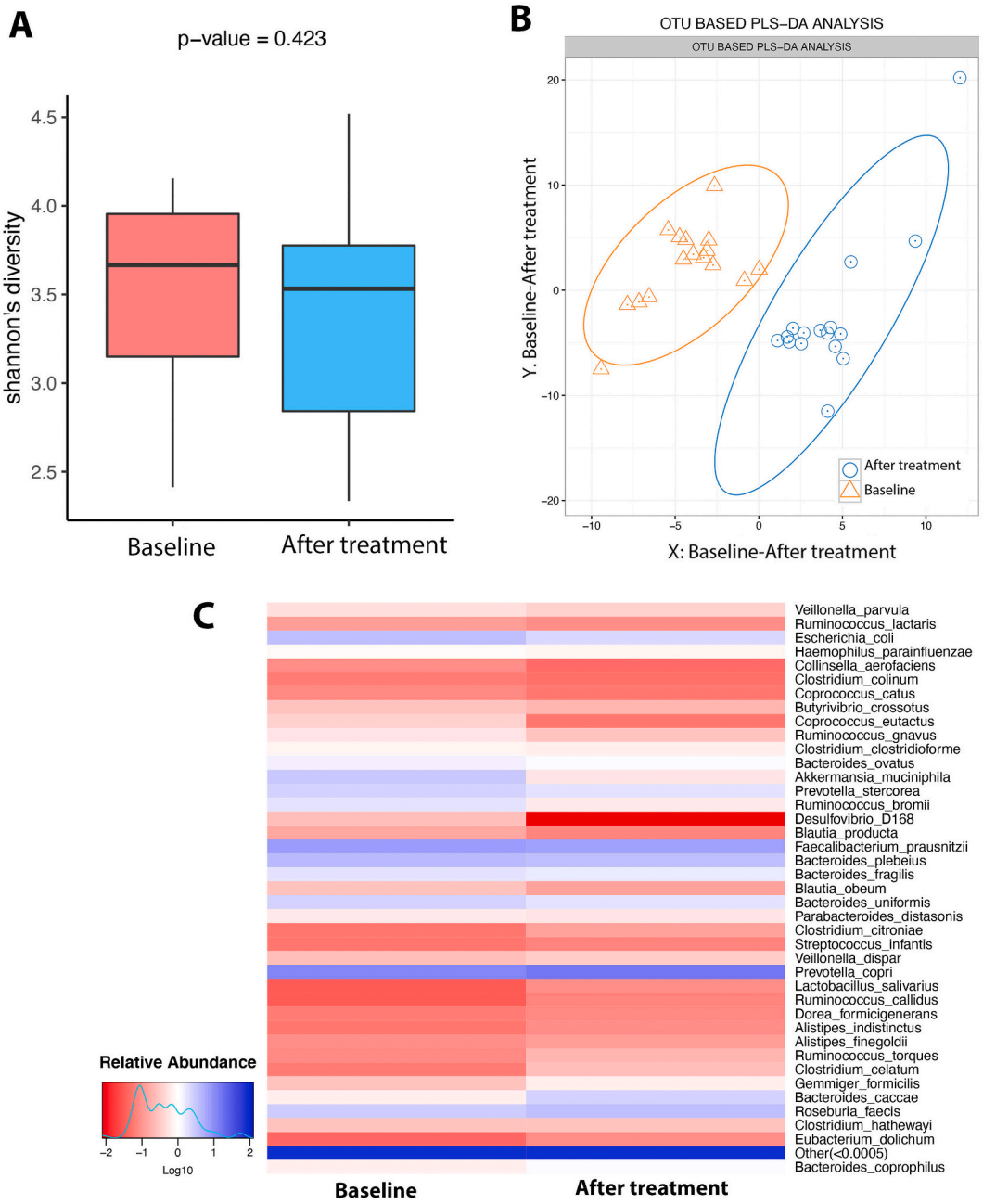

Fig. 1. Analysis of fecal microbiota between baseline and after treatment samples in patients with esophageal squamous cell carcinoma. (A) Shannon index of alpha diversity. (B) Partial least squares discriminant analysis plot of unweighted Unifrac distances. (C) Relative abundance of the main species in the feces.

gastrointestinal reactions and acute kidney injury were observed in the remaining two patients, respectively. Based on radiographic assessment of the best therapeutic efficacy according to RECIST 1.1, 7 patients achieved partial response (PR), 18 had stable disease (SD), and 6 had disease progression (PD). We grouped the responders (R) from the non-responders (NR) for further analysis; patients included in the R group achieved PR and SD, whereas those in the NR group achieved a PD response. The baseline characteristics of the patients are summarized in Table 1.

Baseline fecal samples were collected from all 31 patients before chemotherapy. A second collection of fecal samples was obtained from 14 patients. A total of 7,224,168 tags were obtained from the 45 samples.

3.2. Gut microbiota composition and association with clinical stage

We compared the alpha diversity of the baseline microbiota of patients with locally advanced and distant metastatic diseases using the Shannon index, which indicated no difference between the groups (Fig. S1 A, $P = 0.37$). The composition of the bacterial community was not significantly different between locally advanced and metastatic cancer cases (Fig. S1 B, ANOSIM $R = -0.05$, $P = 0.992$). Based on the relative abundance at the species level, the top ten species with the highest abundance in both groups were identified as *Fecalibacterium prausnitzii*, *Bacteroides uniformins*, *B. fragilis*, *Parabacteroides distasonis*, *Bacteroides ovatus*, *Eschericha coli*, *Roseburia faecis*, *Bacteroides caccae*, *Ruminococcus bromii,* and *Ruminococcus gnavus* (Fig. S1 C). The abundances of these top 10 taxa were not significantly different between patients with different cancer stages. Nevertheless, the abundance of *Bacteroides acidifaciens* was significantly higher in patients with distant metastases ($P = 0.009$). By contrast, the abundance of *Clostridium colinum* ($P = 0.024$) and *Ruminococcus calidus* ($P = 0.008$) both decreased in patients with distant metastatic disease compared to that in patients with locally advanced stage cancer (Table S1).

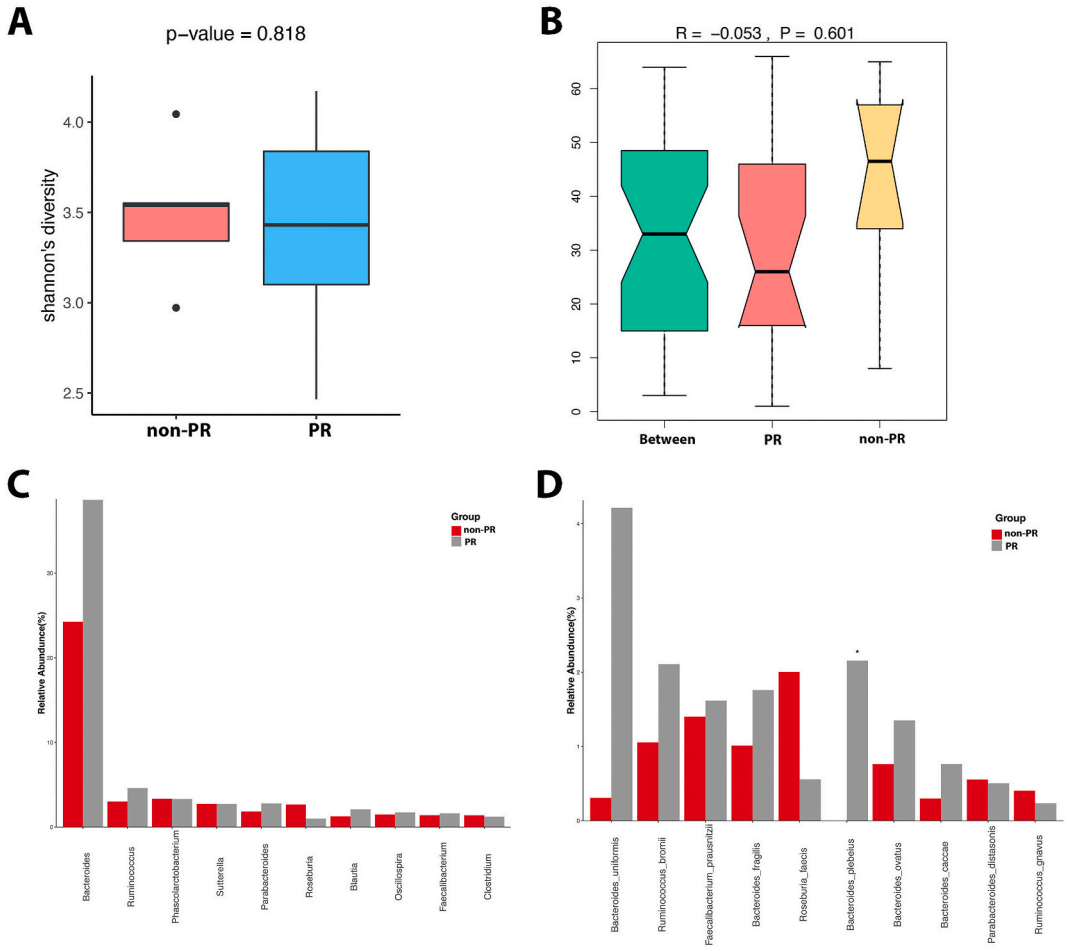

Fig. 2. Analysis of fecal microbiota between the partial response (PR) and non-PR patients with esophageal squamous cell carcinoma. (A) Shannon index of alpha diversity. (B) Partial least squares discriminant analysis plot of unweighted Unifrac distances of the top 10 species with the highest abundance in baseline fecal samples at the genus level (C) and the species level (D).

3.3. Differences in the gut microbiota before and after TP chemotherapy

Of the 31 patients with clinical efficacy data, 14 were sampled from the second fecal sample at the time of efficacy evaluation. We investigated the variation in the gut microbiota because of the TP chemotherapeutic regimen. Therefore, the microbiota diversity was compared between the samples before (baseline) and after chemotherapy (after treatment). No significant differences in alpha diversity were detected (Fig. 1A, $P = 0.423$). Meanwhile, we observed significant differences in microbial composition between the baseline and post-treatment groups at the genus level (Fig. 1B). Furthermore, at the species level, the abundance of *Akkermansia muciniphila* significantly increased (Fig. 1C, $P = 0.013$) in fecal samples after TP chemotherapy, while that of *Pyramidobacter piscolens* decreased (P = 0.043, Table S2).

3.4. Correlations between baseline gut microbiota and chemotherapy efficacy

The objective response rate in patients with ESCC was 22.6 %, whereas the disease control rate was 80.6 %. We grouped the 31 patients into two groups: PR response (n = 7) and non-PR response. We also compared the microbiota of the R and NR groups. Age distribution was balanced between the groups.

No significant differences in the alpha diversity of baseline fecal microbiota were detected between the PR and non-PR groups (Fig. 2A). The ANOSIM analysis based on abundance did not demonstrate significant differences in variation in microbial composition

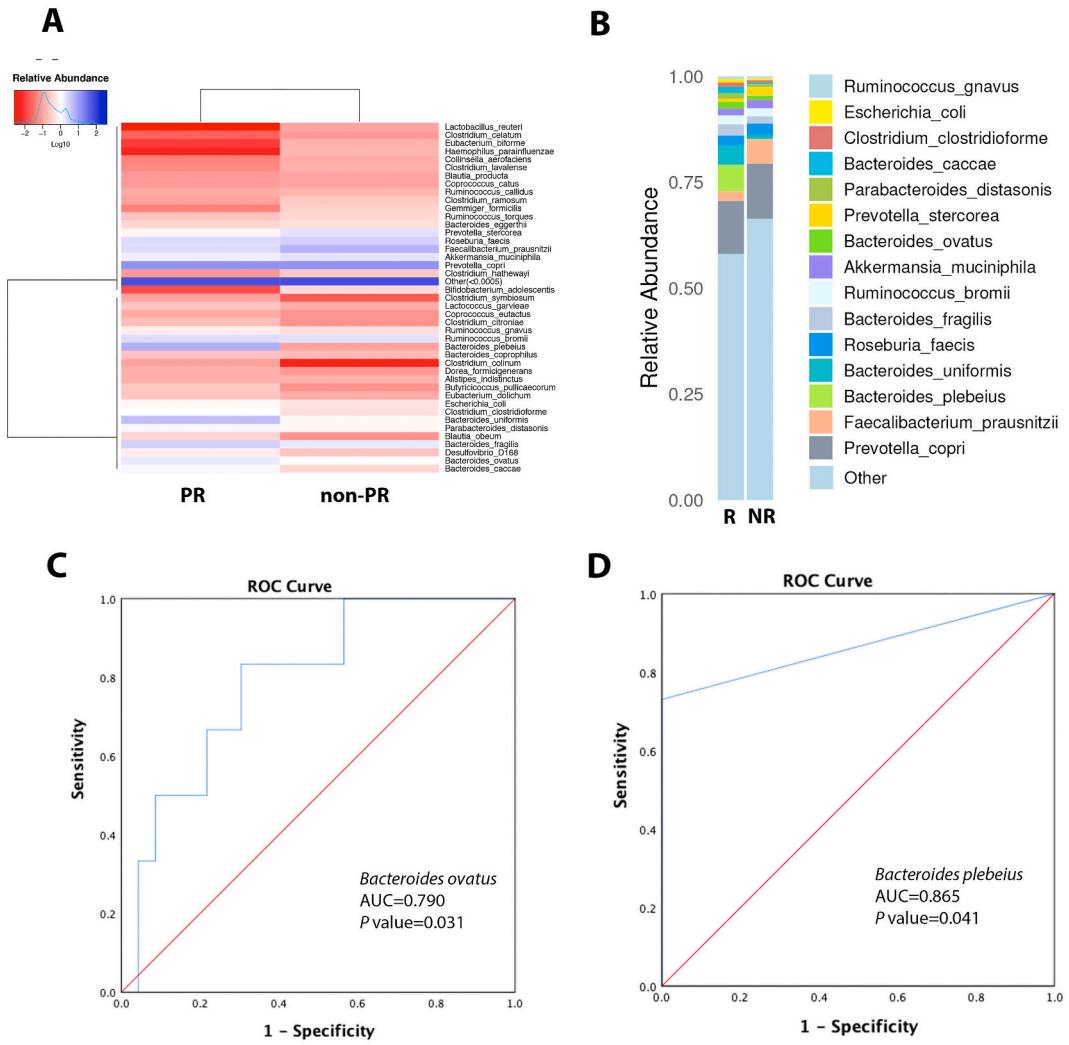

Fig. 3. (A) Relative abundance of the main species in the baseline feces between the partial response (PR) and non-PR groups. (B) Relative abundance of the main species in the baseline feces between the responder (R) and non-responder (NR) groups. (C) Receiver operating characteristic (ROC) curve of *Bacteroides ovatus* for discriminating PR from non-PR (n = 31, area under the curve [AUC] = 0.790, 95 % confidence interval 0.602–0.977, $P = 0.031$). (D) ROC curve of *Bacteroides plebeius* for discriminating the response of the R group from those of the NR group (n = 31, AUC = 0.865, 95 % confidence interval 0.723–0.999, $P = 0.041$).

Table 2
Statistics of ROC analysis of bacterial abundance for distinguishing patients with different chemotherapeutic response.

Efficacy	*P* value									
	Akkermansia muciniphila	Bacteroides fragilis	*B.*[a] *plebeius*	*B.*[a] *ovatus*	*Escherichia coli*	*Faecalibacterium prausnitzii*	*Ruminococcus gnavus*	*R.*[b] *bromii*	Parabacteroides distasonis	*Roseburia faecis*
PR vs non-PR	0.333	0.957	0.374	0.031	0.957	0.306	0.829	0.647	0.374	0.554
R vs NR	0.720	0.943	0.041	0.616	0.943	0.174	0.352	0.197	0.720	0.720

ROC, receiver operating characteristic; PR, partial response; R, responders; NR, non-responders.
[a] B., Bacteroides.
[b] R., Ruminococcus.

between the groups (Fig. 2B). In the relative abundance at the genus (Fig. 2C) and species levels (Fig. 2D), the top 10 taxa with the highest abundance in the baseline samples are presented. At the genus level, the abundances of *Bacteroides, Ruminococcus, Phascolarctobacterium, Sutterlla, Roseburia, Blautia, Oscillospira, Faecalibacterium,* and *Clostridium* were relatively high in all cases and did not differ significantly between the PR and non-PR groups in statistical analysis. To further explore the statistical significance of the comparison between the groups with different efficacies at the species level, we performed the Wilcoxon rank-sum test. *B. plebeius* demonstrated significant differences in abundance between the PR and non-PR groups ($P = 0.043$, Fig. 3A, Table S3). No significant differences were observed among the other species.

We re-analyzed the baseline abundance differences between the R and NR patients. Similarly, no significant differences in the microbial diversity were observed between them. At the species level, the abundance of *B. plebeius* was significantly higher in the R group ($P = 0.045$; Fig. 3B–Table S4). We also observed that the abundance of *Clostridium clostridioforme*, *Bacteroides uniformis*, and *B. caccae* was higher in patients in the R group; however, the differences were not statistically significant (Fig. 3B).

We performed an ROC curve analysis of the abundance of baseline fecal bacteria to identify patients with ESCC receiving TP chemotherapy with different efficacies (PR/non-PR, R/NR). We calculated the optimal cutoff value for the abundance of the top 20 species to identify the specific bacteria that could be determined as predictors of efficacy. The results revealed that only the abundance of *B. ovatus* may be a predictor for distinguishing patients with PR from those without PR, with a sensitivity of 83.3 % and specificity of 69.6 %. The statistical values of the top 10 most abundant taxa are listed in Table 2. As demonstrated in Fig. 3C, the optimal cut-off value of the abundance of *B. ovatus* was 0.4609, which indicate a significantly better response to PR in patients with a baseline abundance of >0.469 ($P = 0.031$, area under the curve (AUC) = 0.790, 95 % confidence interval (CI) 0.602–0.977). Furthermore, the ROC curve analysis suggested that the abundance of *B. plebeius* was positively associated with the response to PR + SD (R) (Fig. 3D). No significant differences in the abundance of other species were observed when distinguishing R from NR (Table 2). The optimal cut-off value of the relative abundance of *B. plebeius* was 0.0019, corresponding to the maximum sensitivity and specificity (73.1 % and 100 %, respectively) for predicting responders (R) in the ROC analysis. The AUC was 0.865 for predicting responders (R) (95 % CI 0.723–0.999, $P = 0.041$).

3.5. Prognostic analysis of the baseline gut microbiota

The median progression-free survival of all the patients was 9 months (95 % CI 6.375–11.625), and the median overall survival was 13 months (95 % CI 5.670–20.330). We analyzed the 20 taxa with the highest levels of abundance to determine whether the baseline abundances of these species were of prognostic significance for ESCC. The univariate analysis revealed that none of these species were significant prognostic factors for PFS or OS (Table 3).

3.6. Correlations between baseline gut microbiota and chemotherapy toxicity

We analyzed the differences in baseline abundance between groups with different severities of chemotherapeutic toxicity. According to the NCI Common Terminology Criteria for Adverse Events, we divided the patients into grades (G) 1–2 and G3–4 into two groups. The ROC analysis was used to determine the relative abundance of baseline gut bacteria to identify patients with ESCC receiving TP chemotherapy with different grades of toxicities (G1–2/G3–4). We calculated the optimal cutoff value for the abundance

Table 3
Univariate analysis of PFS and OS in ESCC cohort.

Variables (gut microbiota)	PFS			OS		
	HR	95%CI	P	HR	95%CI	P
Akkermansia muciniphila	1.023	0.923–1.135	0.669	1.020	0.908–1.145	0.741
Bacteroides caccae	0.904	0.682–1.197	0.480	0.850	0.601–1.204	0.360
Bacteroides fragilis	0.927	0.781–1.100	0.385	0.969	0.812–1.158	0.732
Bacteroides ovatus	0.968	0.728–1.288	0.826	1.057	0.783–1.428	0.716
Bacteroides plebeius	0.994	0.934–1.058	0.857	1.000	0.937–1.067	0.996
Bacteroides uniformis	0.959	0.820–1.122	0.599	0.962	0.814–1.136	0.645
Bifidobacterium adolescentis	0.051	0–110.824	0.448	0.117	0.000–134.2	0.550
Blautia obeum	0.706	0.220–2.260	0.558	0.751	0.226–2.490	0.639
Blautia producta	1.354	0.331–5.531	0.673	1.379	0.323–5.893	0.664
Clostridium clostridioforme	0.733	0.363–1.477	0.384	0.741	0.357–1.539	0.421
Clostridium colinum	0.441	0.009–22.56	0.684	0.507	0.008–31.32	0.747
Clostridium hathewayi	0.774	0.263–2.282	0.642	0.773	0.245–2.435	0.660
Escherichia coli	1.003	0.965–1.042	0.882	0.998	0.950–1.049	0.940
Faecalibacterium prausnitzii	0.981	0.927–1.039	0.516	0.983	0.926–1.042	0.557
Gemmiger formicilis	0.587	0.126–2.732	0.498	0.571	0.109–3.001	0.508
Parabacteroides distasonis	0.954	0.746–1.220	0.710	0.971	0.756–1.248	0.820
Prevotella corpi	1.016	0.988–1.045	0.260	0.940	0.948–1.509	0.550
Roseburia faecis	0.965	0.811–1.148	0.687	0.967	0.804–1.164	0.725
Ruminococcus bromii	1.154	0.966–1.378	0.114	1.063	0.894–1.264	0.487
Ruminococcus gravus	0.981	0.598–1.609	0.940	1.011	0.607–1.685	0.966

PFS, progression-free survival; OS, overall survival; ESCC, esophageal squamous cell carcinoma.

Table 4
Statistics of ROC analysis of bacterial abundance for distinguishing patients with different chemotherapeutic toxicity.

Adverse Events (Toxicity)	*P* value									
	Akkermansia muciniphila	Bacteroides uniformis	*B.*[a] *plebeius*	*B.*[a] *ovatus*	*Escherichia coli*	*Faecalibacterium prausnitzii*	*Ruminococcus gnavus*	*R.*[b] *bromii*	*Bifidobacterium adolescentis*	*Roseburia faecis*
G1-2 vs G3-4	0.919	0.028	0.049	0.059	0.683	0.575	0.838	0.079	0.203	0.541

ROC, receiver operating characteristic; G garde of adverse Events.
[a] B., Bacteroides.
[b] R., Ruminococcus.

of the top 20 species to identify the bacteria that could be used as predictors of more severe adverse reactions. The statistical values of the top 10 most abundant taxa are listed in Table 4.

The results revealed that the abundance of *B. plebeius* may be a predictor for identifying patients with grade 3–4 toxicities after chemotherapy. The sensitivity was 85.7 % and the specificity was 63.6 % (Fig. 4A). The optimal cutoff value of the abundance of *B. plebeius* was 1.4995, which revealed that a significantly more severe toxicity in patients with a baseline abundance of >1.4995 ($P = 0.049$, AUC = 0.750, 95%CI 0.529–0.971). Moreover, *B. uniform* was also identified as a predictor of higher-grade adverse reactions. As presented in Fig. 4B, the cut-off value of the abundance of *B. uniform* was 1.1428, which suggests a significantly worse toxicity (G3–4) in patients with a baseline abundance of >1.1428 ($P = 0.028$, AUC = 0.779, 95 % CI 0.609–0.949). The sensitivity was 85.7 % and the specificity was 72.7 %.

Based on the ROC analysis of the predictors of G1–2/G3–4 toxicities in all patients with ESCC, we constructed our multi-parameter predicting models with a combination of these two significant species to explore better biomarkers for predicting chemotherapy toxicity. The sensitivity and specificity of the model demonstrated a better predictive ability than a single bacteria (*B. uniform* or *B. plebeius*). The model demonstrated a better ability to distinguish G1–2 from G3–4 adverse reactions in patients with ESCC receiving the TP regimen (AUC = 0.825, $P = 0.011$, Fig. 4C). The sensitivity was 85.7 % and the specificity was 77.3 %.

4. Discussion

The features of the microbiota are less well-investigated in ESCC than in esophageal adenocarcinoma [30]. A study revealed that *Erysipelotrichales* and *Clostridiales* were enriched in the gastric corpus microbiota of patients with ESCC compared to that in healthy controls [31]. *Prevotella* spp., *E. coli, B. fragilis* and *Clostridium* spp. were significantly increased in ESCC tumor tissues [32], whereas *Bacteroidetes* and *Fusobacteria* were decreased in patients with ESCC compared with healthy individuals [33]. The specific bacterium *Porphyromonas gingivalis* in the esophageal mucosa was also considered a biomarker of ESCC, which positively associated with the severity of ESCC and worse prognosis [34]. Currently, more evidence is available regarding the characteristics of the EC tissue bacteria in EC. However, the role of the gut microbiota in patients with ESCC remains unclear. Our study revealed the features and spectrum of the gut microbiota in patients with ESCC, which may establish a microbial signature for predicting the clinical outcomes of ESCC.

In our study, the relative abundance of fecal *B. acidifaciens* was significantly increased in patients with distant metastases, whereas that of *C. colinum* and *R. calidus* was negatively correlated with a more advanced stage. *Bacteroides* is a dominant genus in the gut and was increased in colorectal cancer compared with healthy people [35], and some species in this genus were positively associated with cancer progression [36]. Similar characteristics of *Bacteroides* were first demonstrated in ESCC, suggesting that a common mechanism exists for bacteria affecting the CRC microenvironment, which requires further study. *Clostridia* are capable of activating intracellular signaling pathways [37], and a previous study has reported a decreased relative abundance of fecal *Clostridia* in patients with CRC compared with that in healthy controls [38]. *Clostridium* species are commensal bacteria in the human gut and are a heterogeneous group of bacteria that can be classified into several clusters with different functions. Fecal *C. colinum* levels are lower in patients with ulcerative colitis (UC) than in healthy controls [39]. Although the impact of *C. colinum* on cancer is unclear, a consensus on a possible correlation between inflammation and malignancy has been suggested. Thus, the impact of *C. colinum* on UC may occur in cancer. Gut *C. colinum* decreased in ESCC with distant metastasis in our study, partly indicating its protective effect, which is consistent with previous studies. Several species from the *Ruminococcaceae* family have been identified as positive prognostic factors in immunotherapy for many cancers because of the involvement of these taxa in secondary bile acid production and metabolism, which may reduce gut inflammation and benefit the immune system [40]. Further studies have revealed that anti-tumor immunity was enhanced by the increased abundance of the *Ruminococcaceae* family, accompanied by an increase in infiltrating IFN-γ+CD8$^+$ T cells in tumor

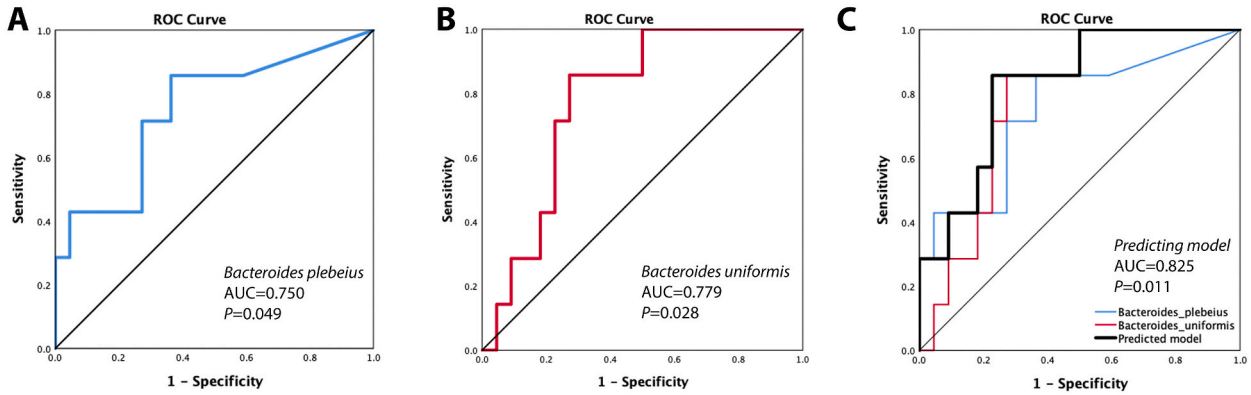

Fig. 4. ROC curve discriminating grade 3–4 toxicities vs. grade 1–2 toxicities after chemotherapy. (A) Performance of *Bacteroides plebeius* in classifying different grades of toxicity (n = 31, AUC = 0.750, 95 % confidence interval 0.529–0.971, $P = 0.049$). (B) Performance of *Bacteroides uniform* in classifying different grades of toxicity (n = 31, AUC = 0.779, 95 % confidence interval 0.609–0.949, $P = 0.028$). (C) Performance of *Bacteroides plebeius, Bacteroides uniform,* and predicted model (n = 31, AUC = 0.825, 95 % confidence interval 0.664–0.986, $P = 0.011$) in classifying grade 3–4 toxicities vs. grade 1–2 chemotherapeutic toxicities.

[41]. Fecal *Ruminococcus callidus* was significantly enriched in patients with favorable clinical response to immunotherapy for hepatobiliary cancers [42]. In our study, the reduction in the abundance of gut *R. callidus* in patients with ESCC with distant metastases demonstrated the protective effects of this taxon.

Pharmacomicrobiomics is a promising approach for investigating the effect of drugs to explore microbial biomarkers for predicting chemotherapeutic responses. By detecting differences in fecal bacteria between baseline and post-treatment samples, we analyzed chemotherapy-associated alterations in the gut microbiota. Reduced diversity has been linked to the use of antibiotics and chemotherapy [43,44]. However, we failed to detect a change in microbial alpha diversity after chemotherapy, which is inconsistent with the results of a previous study [44]. Immunotherapy was first approved by the CFDA in December 2020. When enrollment began in 2018, the clinicians mainly selected chemotherapy. Although the paclitaxel-based regimen was not preferred by the National Comprehensive Cancer Network as a first-line choice, it is commonly used in locally advanced disease and has demonstrated a good response in metastatic disease. Paclitaxel and cisplatin combination regimens have been extensively applied in China and have exhibited satisfactory responses in real-world studies. Moreover, the Guidelines of Chinese Society of Clinical Oncology recommend TP for grade I. Thus, TP was selected as the preferred treatment regimen in the present study. Studies have suggested that cisplatin damages the gut epithelial barrier and induces bacterial translocation into the blood circulation [18]. Limited studies have investigated the effects of paclitaxel on the gut microbiome.

In our study, the paired analysis of bacterial composition and abundance variation demonstrated opposite trends for the two species. *Akkermansia muciniphila* (*A. muciniphila*), a commensal gram-negative anaerobe, accounts for 1%–4% of the gut microbiome [45]. Several studies investigating the effects of *A. muciniphila* in colorectal cancer have yielded inconsistent results. An increased abundance of gut *A. muciniphila* is associated with the prevention of CRC [46]; however, it has also been observed to be negatively correlated with the prognosis of CRC in some animal studies [47]. *P. piscolens* was first isolated from the human oral cavity and has been associated with oral dysbiosis [48]. A previous study has reported that gut *P. piscolens* was associated with an increased risk of CRC progression [49]. One study has reported a decrease in the relative abundance of fecal *Firmicutes* and an increase in *Proteobacteria* post-chemotherapy. However, evidence on the alteration of *A. muciniphila* or *P. piscolens* post-treatment is limited. The second fecal sampling was performed 6 weeks after chemotherapy, the time point at which the gut microbiome recovers from the instant effect of chemotherapeutic drugs. Host immune regulation may play a role in maintaining the balance in the microenvironment. These alterations most likely suggest the long-term effects of the drugs, which may demonstrate a microbial variation trend over several cycles of chemotherapy. These chemotherapy-induced microbial changes indicate that the increased risk of infection may be due to microbial imbalance and that chemotherapeutic toxicity and anti-tumor responses might also be mediated by microbial alteration.

Two significantly enriched taxa in the *Bacteroidetes* phylum between the PR and non-PR groups and the R and NR groups were associated with favorable outcomes of TP chemotherapy in our study. In previous studies, several commensal strains that produce short-chain fatty acids such as butyrate were identified as positive predictors of a favorable response to immunotherapy. Evidence indicates that fatty acids can induce immune responses and improve epithelial barrier function by regulating T cell differentiation [50]. Studies have demonstrated that several species in the phylum *Firmicutes* are associated with favorable responses and survival benefits to immunotherapy in hepatobiliary cancer [42], melanoma [21], lung cancer, and renal cell carcinoma [22]. Nevertheless, bacterial taxa in the phylum *Bacteroidetes* are commonly observed to have a heterogeneous impact on immunotherapy [42]. The controversial impact of *Bacteroidetes* on cancer immunotherapy may be attributed to the features of specific species and different kinds of cancers. Accumulating evidence has demonstrated the impact of gut microbiota on solid tumor with anti-tumor immunotherapy. The gut microbiome is known to affect metabolism and immune responses to modify the anti-tumor activity of immunotherapies. Compared to immunotherapy, much less evidence has been provided concerning the impact of the gut microbiota on traditional chemotherapy.

The therapeutic response of cisplatin in germ-free animals with lung cancer is attenuated by the use of antibiotics, suggesting that the bacterial profile may affect the chemotherapy response [51]. We observed that patients with relatively high abundance of *B. plebeius* and *B. ovatus* were more likely to experience better chemotherapeutic responses. A previous study identified a positive correlation between *B. ovatus/xylanisolvens* and cytotoxicity or targeted chemotherapy efficacy in mice with lung cancer [52], which is partly consistent with the present study. However, Zhang et al. [53] examined the gut microbiota of 60 patients with lung cancer and reported that the abundance of *Bacteroides* was similar in patients with stable disease and disease progression. *Bacteroides* is one of the top ten high abundance genera in the human gut [7,8]; thus, we deduced that different levels of this genus might affect the response to cancer treatment. Nevertheless, the characteristics of microbial predictive significance during chemotherapy are not consistent and may be organ-specific. Different combinations of chemotherapeutic drugs may be responsible for this effect. The mechanisms of the ability of *Bacteroides* to modulate chemotherapeutic responses have not yet been identified. Moreover, some studies have reported that *F. nucleatum* was related to cancer-specific survival in ESCC [16]. We did not detect any bacteria that affected the survival outcomes. In addition to the gut microbiome, several other factors may be implicated in the survival of patients with cancer. Therefore, comprehensive research is needed for further exploration.

Similar to previous studies [25,26,54], our study revealed that a high abundance of specific species was associated with severe drug toxicity. We demonstrated that *B. plebeius* and *B. uniform* may modulate the side effects of the TP regimen. Studies have demonstrated that increases in *Fusobacteria* and *Proteobacteria* correlate with drug-induced gastrointestinal toxicity [54]. Results obtained from another study demonstrated that *K. pneumoniae* was frequently detected in patients with chemotherapy-induced diarrhea [25]. *Enterobacteriaceae* have been identified as promoters of mucositis [26]. To our knowledge, *Bacteroides plebeius* has been identified as a CRC-associated Bacterium in several studies [55,56], suggesting that it either promotes cancer development or is favored in the cancerous state. Thus, the mechanisms of enhanced efficacy and toxicity described in the current study cannot be simply interpreted.

Several microbiome-related studies have provided important insights into the prognostic and predictive significances of oncomicrobiomes. The most critical issue is the use of the gut microbiome to improve the therapeutic response and prognosis of patients with

cancer. Therapeutic options include FMT and microbiota-derived metabolic products. Currently, data regarding the application of FMT in specific anti-tumor therapies are insufficient. Oral administration of FMT to a mouse model during oxaliplatin and fluorouracil combined chemotherapy led to less severe mucositis and diarrhea [57]; however, the efficacy of chemotherapy was not evaluated. FMT from patients with better efficacy in germ-free mice significantly improved immunotherapy response [22]. Thus, probiotics, prebiotics, and synbiotics are considered approaches for manipulating the microbiota. Therefore, adoption of these strategies should be carefully assessed. Safety and validity require further study, owing to previous investigations being performed mostly in animal models. Concerning some bacteria with predictive ability for both better efficacy and severe toxicity of chemotherapy identified in the current study, developing a strategy for preparing optimal FMT is especially difficult. Moreover, each patient has an individual microbiota composition, which makes obtaining a homogenous FMT product difficult. Although some issues should be considered, the microbiome-based strategy is encouraging for the improvement of cancer therapy.

Our study has some limitations. The sample size is not big enough, especially in subgroups based on the efficacy. Furthermore, the gut microbiome can be affected by numerous factors, such as diet, nutritional status, age, and physical activity. Therefore, heterogeneity requires further investigation, considering these complex confounding factors. Third, the number of fecal samples collected after chemotherapy was insufficient to assess the association between microbial changes and treatment outcomes. Finally, we did not use animal models to identify the mechanisms underlying the impact of the microbiota on chemotherapeutic responses.

5. Conclusions

Our study observed an association between the abundance of specific bacteria and chemotherapeutic response to the TP regimen in patient swith ESCC. *B. plebeius* and *B. ovatus* were identified as predictors to identify patients with better efficacy to TP chemotherapy. Moreover, B. plebeius and B. uniform may be indicators to identify patients with grade 3–4 toxicity after chemotherapy. Our study provides a theoretical basis for the application of a strategy based on the microbiome of patients with ESCC. Thus, further exploration is needed to assess individual gut microbial features to predict the efficacy and toxicity of cancer therapy.

Data availability statement

Data included in article/supp. material/referenced in article.

Ethics statements

The study have been approved by the ethics committee of Peking Union Medical Hospital (JS-2745). The patients provided their written informed consent to participate in this study.

CRediT authorship contribution statement

Ningning Li: Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Liwei Gao:** Writing – review & editing, Methodology, Data curation. **Yuping Ge:** Software, Methodology, Formal analysis. **Lin Zhao:** Writing – review & editing, Visualization, Methodology. **Yingyi Wang:** Writing – review & editing, Supervision, Conceptualization. **Chunmei Bai:** Writing – review & editing, Supervision, Methodology.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Nomenclature

ESCC	esophageal squamous cell carcinoma
EC	esophageal cancer
SCC	squamous cell carcinoma
PFS	progression-free survival
OS	overall survival
CI	confidence interval
ROC	receiver operating characteristic
AUC	area under the curve
SD	stable disease
PD	disease progression

R	responders
NR	non-responders
PR	partial response
ANOSIM	analysis of similarity
CRC	colorectal cancer

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e32770.

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