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Original Article

Changes in *Bacteroides* and the microbiota in patients with obstructed colorectal cancer: retrospective cohort study

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Abstract

Background: The relationship between intestinal obstruction due to colorectal cancer (CRC) and the gut microbiota remains largely unknown. The aim of this study was to investigate the potential association between alterations in gut microbiota and CRC in the presence of intestinal obstruction.

Methods: Patients with CRC with or without obstruction were recruited and compared using 1:1 propensity score matching (PSM). Total DNA from tumours and adjacent normal tissues of 84 patients and 36 frozen tumour tissues was extracted and amplified. 16S RNA sequencing was used to uncover differences in microbiota composition between the two groups.

Results: A total of 313 patients with CRC were recruited. Survival analysis demonstrated that patients in the obstruction group had shorter overall survival time and disease-free survival (DFS) time than those in the non-obstruction group. Microbial richness and diversity in tumour tissues of patients with obstruction were significantly higher than those of patients with no obstruction. The alpha diversity indices and beta diversity exhibited were different between the two groups ($P < 0.05$). At the phylum and genus levels, Bacteroidetes were significantly enriched in the tumour tissues of patients with obstruction. Alpha diversity in tumour tissues was closely related to specific microbiota. These findings were replicated in the 16S rRNA analyses from frozen samples. There were more Bacteroidetes in CRC patients with obstruction.

Conclusions: Patients with obstructed CRC have worse prognosis and have differences in their microbiota. Higher levels of *Bacteroides* were observed in patients with obstructed CRC.

Introduction

Approximately 20 per cent of patients with colorectal cancer (CRC) present with intestinal obstruction as the initial presentation. Patients presenting with obstruction have a considerably poorer clinical prognosis, with 5-year survival rates ranging from 31 per cent to 42 per cent[1–3]. Studies have demonstrated that gut microbiota plays a vital role in the development of CRC[4,5]. Over 100 trillion micro-organisms are present in the gastrointestinal tract of hosts, aiding in the maintenance of tissue homeostasis and influencing intestinal nutrition and metabolism. Gut microbiota can exert both beneficial and detrimental effects via metabolites or interactions with host intestinal epithelial cells[6,7]. Disruption to intestinal homeostasis can lead to pathology, including inflammatory bowel diseases, adenomas and cancer[5,8].

Bacteroidetes is a prominent bacterial lineage that evolved early in the evolutionary process, and related studies have demonstrated that Bacteroidetes play a crucial role in the development of CRC[9–11]. The primary components of gut microbiota are strict anaerobes, with Bacteroidetes and Actinobacteria comprising over 90 per cent of bacterial phyla in the colon[12]. Animal studies have demonstrated that gut microbiota are critical in mediating the carcinogenic effects of 1,2-dimethylhydrazine in conventional rats, and specific species of *Bacteroides* could promote colorectal carcinogenesis by increasing aberrant crypt foci. Transferring stool from CRC patients to germ-free mice resulted in enhanced intestinal cell proliferation

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and promoted tumour formation[13,14]. Abdulamir *et al*. reported that NF-κB and IL-8 are key mediators in the development of gallbladder *Streptococcus*-related carcinoma, progressing from adenoma to carcinoma[15]. Enrichment of *Fusobacterium nucleatum* in CRC tissues is associated with a shorter survival time and may serve as a biomarker for poor prognosis[16].

The relationship between CRC complicated with intestinal obstruction and specific gut microbiota remains unexplored. Questions include whether changes in intestinal specific flora are associated with intestinal obstruction, how gut microbiota changes in patients with CRC after intestinal obstruction, and whether specific gut microbiota affect the prognosis of CRC patients with obstruction.

The aim of this study was to investigate whether differences in specific gut microbiota were present in patients with intestinal obstruction secondary to CRC.

Materials and methods

Patient selection

Patients with pathologically confirmed CRC who underwent surgical resection at Wuhan Union Hospital between 2015 and 2019 were recruited. The inclusion criteria were patients diagnosed with primary CRC with or without obstruction, confirmed by pathological report, and complete clinicopathological and follow-up data. Exclusion criteria were active infection, active inflammatory bowel disease (IBD), intestinal fibrosis, organ transplantation, oral medications over the past month (including proton pump inhibitors, probiotics and alcohol), received chemotherapy, radiation, or antibiotics within one week prior to surgery, or insufficient tissue excised for 16S rRNA sequencing. Complete intestinal obstruction was defined as the inability of bowel contents to pass through due to an intestinal tumour, accompanied by abdominal distension and pain. Radiological findings on abdominal standing X-ray plain film included air levels, gas shadowing, and signs of intestinal obstruction in the upper abdomen. Abdominal CT findings included stenosis of the intestinal segment, proximal bowel dilatation, and collapse of the distal end. Colonoscopy findings included inability to pass through the intestinal stenosis. This study was approved by the Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology (No. 2021–0793), and all subjects provided written informed consent before participating in the study. To verify the dependability of 16S rRNA sequencing on paraffin-embedded tissues, fresh tissues were collected from 36 recently enrolled CRC patients who met the same criteria in March 2023 for 16S rRNA sequencing analysis. Paraffin-embedded tissues were collected from these same patients for subsequent microbial fluorescence *in situ* hybridization (FISH) analysis to investigate the differences in *Bacteroides* between the two groups. Our study was in line with the Helsinki Declaration II.

Data collection

Clinical information collected included age, gender, BMI, intestinal obstruction, tumour location, tumour differentiation, tumour size, perineural invasion, vascular invasion, T stage, N stage, TNM stage, postoperative chemotherapy, and laboratory data, including liver and renal function. The serum tumour markers carcinoembryonic antigen (CEA), carbohydrate antigen 19–9 (CA19–9), carbohydrate antigen 12–5 (CA125) and carbohydrate antigen 72–4 (CA72–4) were also measured. Tumour staging was classified according to the 8th edition of the American Joint Commission Tumour Staging System, and high-risk patients with stage II or higher received adjuvant radiotherapy or chemotherapy after surgery. Follow-up information was collected, including overall survival (OS), defined as the time between the first day of surgical resection and the date of death or last visit, and disease-free survival (DFS), defined as the time between the first day of surgical resection and the date of death, any type of tumour progression, recurrence, or last visit.

Propensity score matching analysis

The included patients were strictly matched 1:1 between the obstruction and non-obstruction groups using the nearest-neighbour algorithm. Age at diagnosis, sex, BMI, TNM stage, histological grade, tumour size, tumour site, and chemotherapy were matched to adjust for confounding indicators and facilitate a balanced comparison between the two groups. The inverse probability of the treatment-weighted algorithm was then applied to further eliminate potential imbalances between the obstruction and non-obstruction groups. The Cox proportional hazards model was used for survival analysis of significant univariable indicators. Sensitivity analyses were performed in both the primary and propensity score-matched cohorts to further validate the conclusions of the univariable Cox analysis.

DNA extraction, PCR amplification, and MiSeq sequencing

Genomic DNA was extracted from CRC tissue samples using the Omega Magobind Soil DNA Kit (Omega Bio-Tek, Norcross, GA, USA), and the resulting DNA was quantified using a UV spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The concentration and purity of the DNA were then assessed using 0.8 per cent agarose gel electrophoresis, and all extracted DNA samples were stored at −80°C until further processing. A series of primers targeting the V3–V4 variable region of the 16S rRNA gene were used to amplify the extracted DNA. The forward primer was 5′-ACTCCTACGGGGAGGCAGCA-3′, and the reverse primer was 5′-GGACTACHVGGGTWTCTAAT-3′[17]. PCR amplification was performed using Applied Biosystems R 2720 Polymerase Chain Amplifiers (Applied Biosystems Instruments, Thermo Fisher Scientific, Waltham, MA, USA), and the PCR products were analysed using double agarose gel electrophoresis. The amplicon was then purified and quantified using the Quant IT PicoGreen dsDNA analysis kit (BioTek FLx800 Microplate Reader, Winooski, VT, USA). A DNA Library LT Library Prep Kit was constructed based on TruSeq Nano DNA, and the resulting library was sequenced using the Illumina MiSeq PE300 platform.

Data processing

The raw sequencing data were screened and assessed for quality, and the data-processing procedure involved the following steps: paired sequencing data were filtered using the sliding window method and saved in the FASTQ format for Illumina MiSeq platform; the paired-end reads of each library were overlapped using FLASH version 1.2.71[18]; microbial sequences were identified using QIIME2 software (Quantitative Insights Into Microbial Ecology, V1.8.02)[19]; chimeric sequences were detected and removed using USEARCH (V5.2.2363) and QIIME2 software (V1.8.0), and representative sequences were categorized by searching the Greengenes database (Release 13.84) with default parameters[20]. A raw operational taxonomic unit (OTU) composition table was then created to record the abundance of each OTU in each sample, and its taxonomic classification, with OTUs having <0.001 abundance in all samples discarded.

Table 1 Comparisons of baseline characteristics between the original cohort, matched cohort and weighted cohort

Covariates	Original cohort			Matched cohort			Weighted cohort		
	Non-obstruction	Obstruction	*P*	Non-obstruction	Obstruction	*P*	Non-obstruction	Obstruction	*P*
n	271	42		42	42		41.1	41.7	
Age, years	55.7 (13.0)	61.5 (13.6)	0.008	62.4 (12.9)	61.5 (13.6)	0.761	61.5 (13.2)	61.4 (13.6)	0.958
Gender, male, *n* (%)	156 (57.6)	25 (59.5)	0.943	22 (52.4)	25 (59.5)	0.660	24.2 (58.9)	24.7 (59.3)	0.964
BMI (kg/m^2)	22.6 (2.9)	21.6 (2.6)	0.033	21.9 (2.5)	21.6 (2.6)	0.702	21.8 (2.6)	21.7 (2.6)	0.821
Primary site, *n* (%)			0.094			0.888			0.982
Left colon	144 (53.1)	26 (61.9)		26 (61.9)	26 (61.9)		25.8 (62.8)	25.9 (62.0)	
Right colon	58 (21.4)	3 (7.1)		14 (33.3)	13 (31.0)		12.1 (29.5)	12.9 (30.8)	
Rectum	69 (25.5)	13 (31.0)		2 (4.8)	3 (7.1)		3.2 (7.7)	3.0 (7.2)	
Histological grade, *n* (%)			0.098			0.072			0.640
Well differentiated	73 (26.9)	5 (11.9)		12 (28.6)	5 (11.9)		12.7 (31.0)	4.9 (11.7)	
Moderately	183 (67.5)	35 (83.3)		30 (71.4)	35 (83.3)		26.3 (64.0)	34.9 (83.5)	
Poorly differentiated	15 (5.5)	2 (4.8)		0 (0.0)	2 (4.8)		2.1 (5.0)	2.0 (4.8)	
Tumour size, *n* (%)			0.111			1.000			0.988
<2 cm	16 (5.9)	0 (0.0)		0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)	
2–5 cm	112 (41.3)	23 (54.8)		20 (47.6)	19 (45.2)		22.5 (54.7)	22.9 (54.8)	
≥5 cm	143 (52.8)	19 (45.2)		22 (52.4)	23 (54.8)		18.6 (45.3)	18.9 (45.2)	
T stage, *n* (%)			0.346			1.000			0.481
T1/2	21 (7.7)	1 (2.4)		1 (2.4)	1 (2.4)		2.0 (4.9)	1.0 (2.4)	
T3/4	250 (92.3)	41 (97.6)		41 (97.6)	41 (97.6)		39.1 (95.1)	40.7 (97.6)	
N stage, *n* (%)			0.185			0.899			0.956
N0	135 (53.5)	18 (42.9)		17 (40.5)	18 (42.9)		18.3 (44.6)	18.0 (43.1)	
N1	67 (24.7)	16 (38.1)		18 (42.9)	16 (38.1)		14.5 (35.2)	15.7 (37.7)	
N2	59 (21.8)	8 (19.0)		7 (16.6)	8 (19.0)		8.3 (20.2)	8.0 (19.2)	
M stage, *n* (%)			0.121			1.000			0.976
M0	222 (81.9)	39 (92.9)		39 (92.9)	39 (92.9)		38.1 (92.7)	38.7 (92.8)	
M1	49 (18.1)	3 (7.1)		3 (7.1)	3 (7.1)		3.0 (7.3)	3.0 (7.2)	
TNM stage, *n* (%)			0.446			1.000			0.692
Stage I/II	124 (45.8)	16 (38.1)		17 (40.5)	16 (38.1)		17.2 (41.7)	16.0 (38.3)	
Stage III/IV	147 (54.2)	26 (61.9)		25 (59.5)	26 (61.9)		23.9 (58.3)	25.7 (61.7)	
Chemotherapy, *n* (%)			0.003			0.505			0.892
No	104 (38.4)	27 (64.3)		23 (54.8)	27 (64.3)		25.9 (62.9)	26.7 (64.1)	
Yes	167 (61.6)	15 (35.7)		19 (45.2)	15 (35.7)		15.2 (37.1)	15 (55.9)	
After radiotherapy, *n* (%)			0.382			1.000			0.963
No	251 (92.6)	41 (97.6)		41 (97.6)	41 (97.6)		40.1 (97.5)	40.7 (97.6)	
Yes	20 (7.4)	1 (2.4)		1 (2.4)	1 (2.4)		1.0 (2.5)	1 (2.4)	
Laboratory results									
WBC, $\times 10^9$/l	6.3 (1.7)	6.5 (2.0)	0.709	6.1 (1.5)	6,5 (2.0)	0.488	6.3 (1.5)	6.5 (2.0)	0.721
HGB, g/dl	110.0 (25.8)	109.7 (23.4)	0.940	101.7 (25.9)	109.7 (23.4)	0.142	104.5 (25.1)	109.8 (23.3)	0.783
PLT, $\times 10^9$/l	250.6 (68.4)	245.4 (63.5)	0.720	234.8 (65.6)	245.4 (73.5)	0.543	249.9 (88.1)	245.9 (83.4)	0.783
Albumin, g/l	39.5 (4.9)	36.5 (4.6)	<0.001	37.6 (4.9)	36.5 (4.6)	0.297	38.4 (5.0)	36.5 (4.6)	0.020
TBIL, μmol/l	10.6 (2.7)	12.6 (3.8)	0.547	11.7 (2.9)	12.6 (2.8)	0.604	11.0 (2.8)	12.6 (3.8)	0.234
Creatinine, μmol/l	72.3 (18.5)	70.5 (15.3)	0.547	72.4 (20.1)	70.5 (15.3)	0.633	74.0 (18.3)	70.4 (15.2)	0.195
BUN, mmol/l	4.9 (1.6)	5.5 (1.7)	0.023	5.3 (1.0)	5.5 (1.7)	0.549	5.1 (1.6)	5.5 (1.7)	0.137
LDH, U/l	190.3 (63.1)	250.5 (82.1)	0.272	188.3 (43.3)	205.5 (72.1)	0.426	190.0 (44.5)	205.7 (72.4)	0.451
CEA, μg/l	97.0 (23.2)	62.4 (21.7)	0.810	421.0 (97.5)	62.4 (21.7)	0.322	213.3 (78.6)	62.7 (22.6)	0.402
CA19–9, kU/l	109.7 (63.0)	191.1 (95.5)	0.139	59.9 (22.4)	191.1 (95.5)	0.126	96.7 (23.4)	192.3 (65.9)	0.241
CA125, U/ml	21.4 (14.2)	42.1 (23.3)	0.006	25.1 (14.5)	42.1 (13.3)	0.114	23.0 (10.2)	42.2 (13.4)	0.031
CA72–4, μg/l	10.6 (3.3)	9.8 (3.1)	0.846	9.1 (3.6)	9.8 (3.8)	0.864	10.0 (3.6)	9.9 (3.1)	0.968
OS, months	35.8 (12.7)	30.8 (11.1)	0.077	34.3 (12.0)	30.8 (11.1)	0.360	33.4 (12.7)	30.8 (11.1)	0.400
DFS, months	34.2 (11.7)	29.4 (10.4)	0.096	34.0 (12.1)	29.4 (11.4)	0.224	32.4 (11.8)	29.3 (11.4)	0.318
Death, *n* (%)	42 (15.5)	18 (42.9)	<0.001	5 (11.9)	18 (42.9)	0.003	7.4 (18.2)	18.0 (43.1)	0.001
Recurrence, *n* (%)	48 (17.7)	19 (45.2)	<0.001	5 (11.9)	19 (45.2)	0.002	8.3 (20.3)	19.0 (45.5)	0.001

For all continuous covariates, the mean values and standard deviations are reported. WBC, white blood cells; HGB, haemoglobin; PLT, platelets; TBIL, total bilirubin; BUN, blood urea nitrogen; LDH, lactate dehydrogenase; CEA, carcinoma embryonic antigen; CA19–9, carbohydrate antigen 19–9; CA72–4, carbohydrate antigen; OS, overall survival; DFS, disease-free survival; CA125, cancer antigen 125.

Microbial fluorescence *in situ* hybridization analysis

The tissues were fixed in Carnoy's solution, embedded in paraffin, and cut into 5-mm thick sections for microbial FISH analysis using 16S rRNA-targeted oligonucleotide probes obtained from probe-Base. The sequence of the 'universal bacterial' probe, EUB338 (Cy3 labelled), was 5′-GCT GCC TCC CGT AGG AGT-3′. The sequence of the *Bacteroides*-targeted probe, BCE182 (FITC labelled), was 5′-TGG TCC GTG TCT CAG TAG-3′. The number of bacteria per field was calculated by examining five random fields at 200× magnification using a microscope. A blinded observer evaluated each field and defined negative, low and high abundance of *Bacteroides* as cases with <5, between 5 and 20 and >20 visualized BCE182 probes per field on average, respectively. Other bacteria were noted as positive if they had >5 bacteria per field with the EUB338 probe but were negative with the BCE182 probe.

Immunohistochemistry

Four serial sections of 5 μm per paraffin block for subsequent immunohistochemistry staining were obtained. Initially, these sections were subjected to baking at 60 °C, followed by deparaffinization in xylene and ethanol. After hydration, we

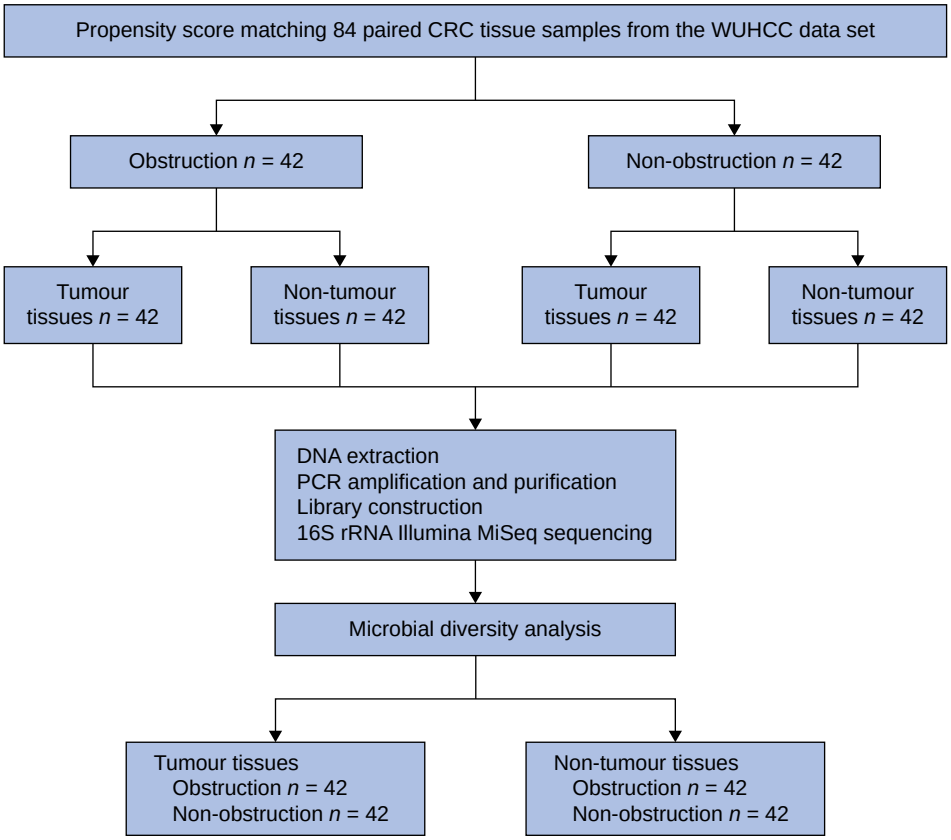

Fig. 1 Study design and flow diagram

CRC, colorectal cancer; WUHCC, Wuhan Union Hospital Cancer Center.

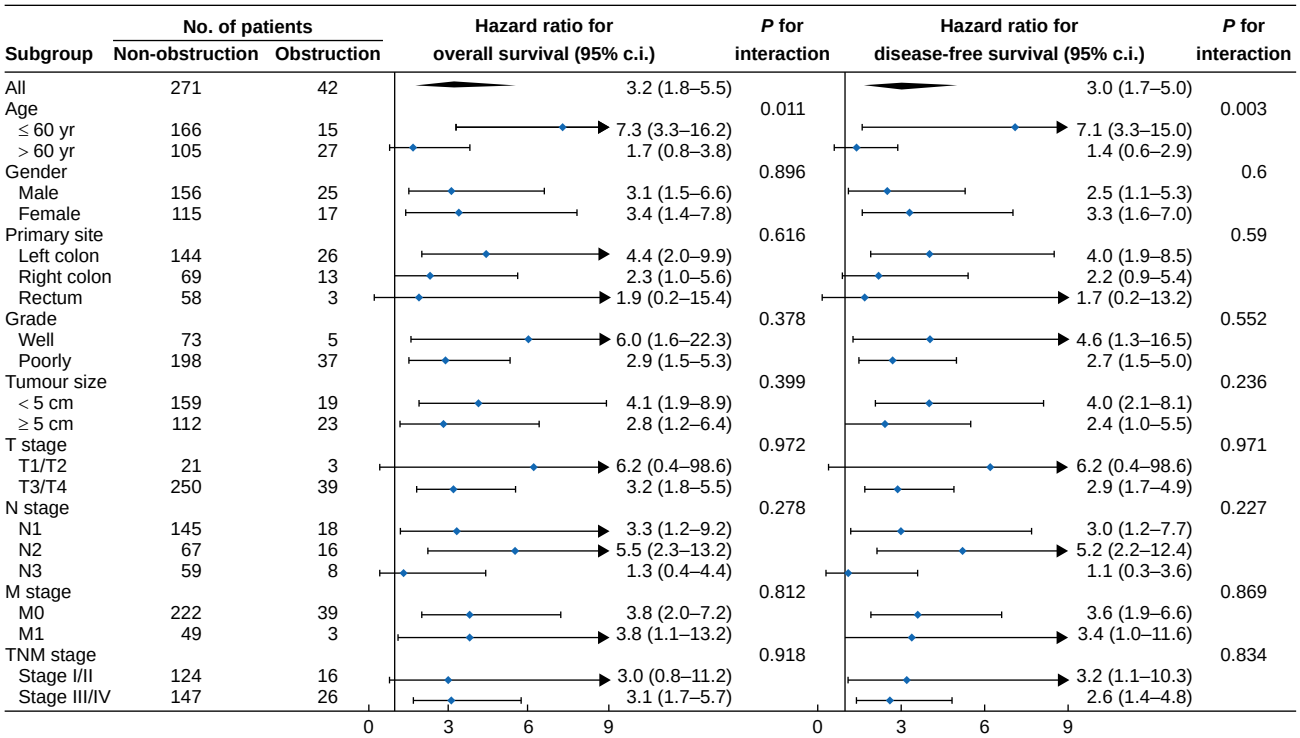

Fig. 2 Subgroup analysis and survival analysis of obstruction in individuals with colorectal cancer

The forest plot revealed the results of subgroup analysis for overall survival and disease-free survival.

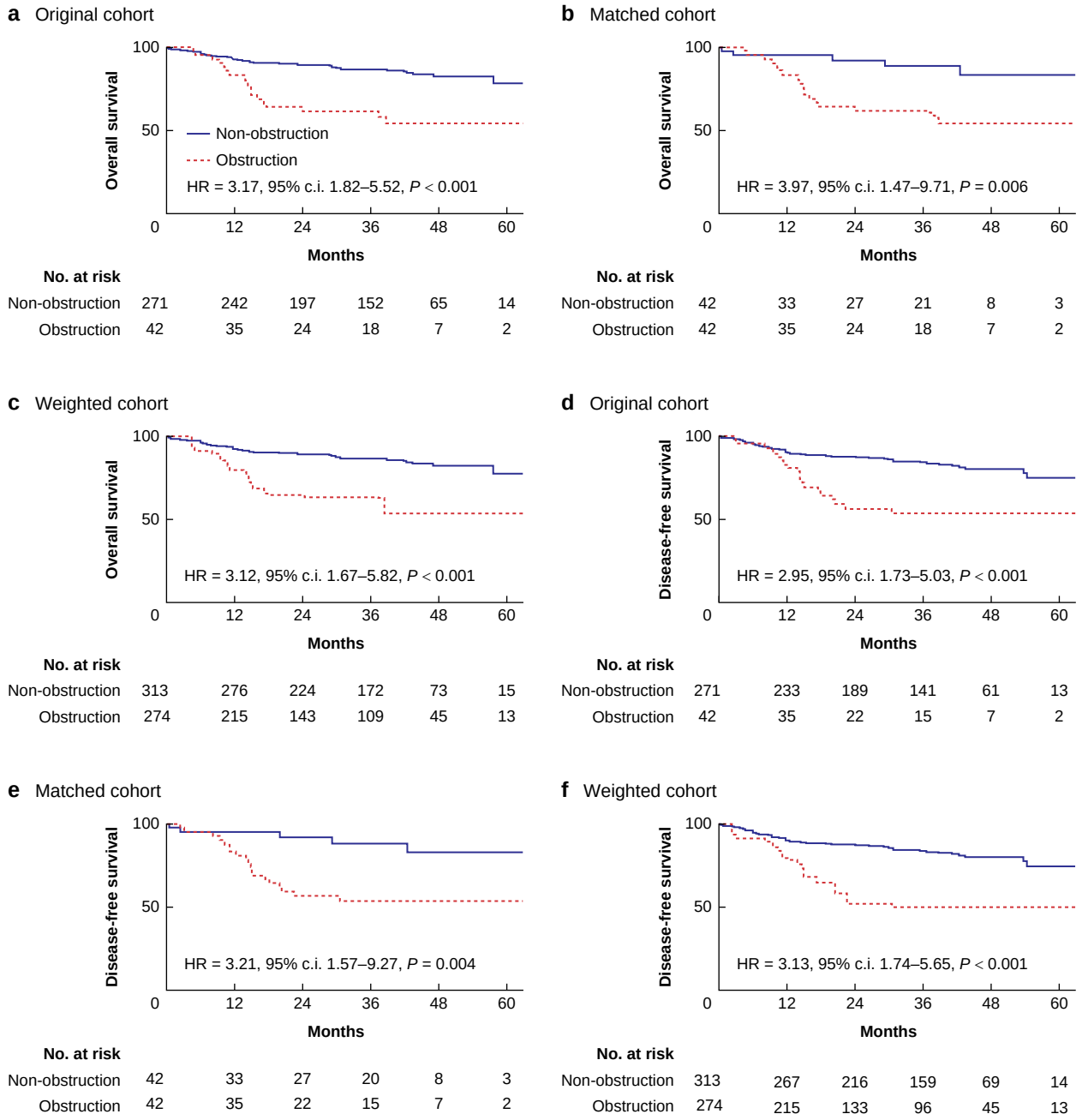

Fig. 3 The prognostic analysis of obstruction and non-obstruction CRC patients after propensity score-matching (PSM)

Kaplan–Meier plots of disease-free survival and overall survival time based on obstruction and non-obstruction groups in the crude cohort (**a**, **d**), PSM cohort (**b**, **e**) and weighted cohort (**c**–**f**). CRC, colorectal cancer.

used 3 per cent hydrogen peroxidase to block endogenous peroxidase activity. For standard antigen retrieval, the sections were heated in a citric acid solution (pH = 6.0) using a pressure boiler. Subsequently, the slides were incubated with primary antibodies [CD4 (ab133616, Abcam, 1:500); CD8 (ab85792, Abcam, 1:400); CD20 (60271-1-Ig, Proteintech, 1:5000); CD68 (ab959, Abcam, 1:6000)] overnight at 4 °C, followed by incubation with a secondary antibody. After staining with 3,3′-diaminobenzidine tetrahydrochloride and counterstaining with haematoxylin, the slides were scanned for further quantitative analysis. The density of CD4+, CD8+, CD20 and CD68 T cells at the invasive margin (IM) was automatically calculated using ImageJ software (version 1.48).

Statistical analysis

All statistical analyses were performed using R software 3.6.2 (R Foundation for Statistical Computing, Vienna, Austria), Graphpad Prism 9 (GraphPad Software, San Diego, California, USA) and SPSS version 24 (Chicago, IL, USA). Continuous data were presented as mean with s.d. and compared by *t*-test or non-parametric test, while categorical indexes were summarized as the frequency with percent and compared by chi-square or Fisher exact test. A Kaplan–Meier survival curve was drawn, and the log-rank test was used to compare the survival time between the obstruction group and the non-obstruction group. $P < 0.05$ was considered statistically significant. The microbial alpha diversity (Chao1, ACE, Shannon,

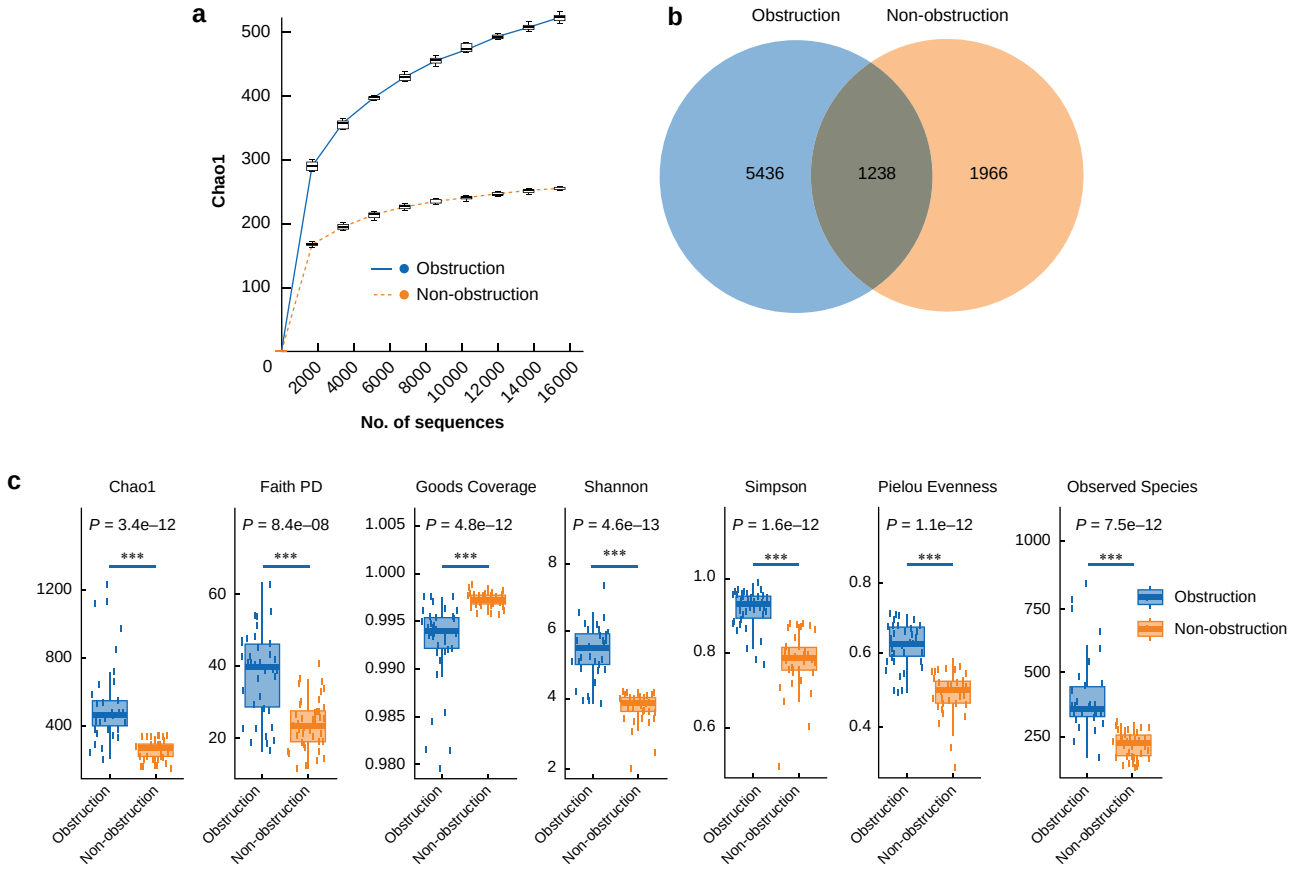

Fig. 4 Rarefaction curve, gut microbiota, and diversity of microbiota composition (continued on next page)

1. Rarefaction curve, gut microbiota and diversity of microbiota composition between obstruction and non-obstruction groups tumour tissues. **a**. Rarefaction curve. **b**. The Venn plots reveal the unique and common taxa between obstruction and non-obstruction groups. **c**. Alpha diversity reveals that species richness was different between the two groups. **d**. Beta diversity analysis revealed by principal coordinate analysis (PCoA). 2. Rarefaction curve, gut microbiota and diversity of microbiota composition between tumour and non-tumour tissues in obstruction. **e**. Rarefaction curve. **f**. The Venn plots reveal the unique and common taxa between obstruction and non-obstruction groups. **g**. Alpha diversity reveals that species richness was different between the two groups. **h**. Beta diversity analysis revealed by PCoA.

Simpson) was compared using a Kruskal–Wallis test[21]. For β diversity, permutation multivariable analysis of variance (PERMANOVA) was used to assess differences in microbial community structure between the two groups, and principal coordinate analysis (PCoA) was performed by weighted UniFrac distance. For the taxonomic composition of microorganisms, we used the Metastats method to compare the microbial differences in tumour tissues between the two groups[22]. The median value was used as the cut-off point to classify the bacterial expression level as 'high' or 'low', and samples with no detectable bacterial expression were classified as negative.

Results

Baseline data characteristics of included patients

A total of 313 patients with CRC who underwent surgical resection met the inclusion criteria, with 271 patients in the non-obstruction group and 42 patients in the obstruction group. There were significant differences between the two groups in terms of age ($P = 0.008$), BMI ($P = 0.033$), chemotherapy ($P = 0.003$), albumin count ($P < 0.0001$), blood urea nitrogen (BUN) ($P = 0.023$), CA125 ($P = 0.006$), mortality ($P < 0.001$) and recurrence ($P < 0.001$) (*Table 1*). To balance these confounding factors, a propensity score matching (PSM) analysis in a 1:1 ratio was performed, and the flowchart of post-PSM screening is illustrated in *Fig. 1*. The correlation between obstruction and clinical metrics in the PSM and weighted cohorts is also presented in *Table 1*.

The prognostic analysis of obstruction and non-obstruction patients with colorectal cancer after propensity score-matching

In the primary CRC cohort, a subgroup analysis was performed to investigate whether the level of obstruction independently impacted the survival of CRC patients who underwent surgical resection. The forest plot demonstrated a significant association between obstruction and unfavourable overall survival (OS) in various subgroups (*Fig. 2*), including age, primary site, T stage, N stage, and TNM stage. Similarly, obstruction was strongly correlated with poorer DFS in most subgroups (*Fig. 2*), such as age, primary site, T stage, and TNM stage. Survival analysis was performed to evaluate the impact of obstruction on the stratification of CRC patients with different survival risks. In the crude population, the HR indicated that the obstruction group had a significantly poorer OS rate (HR = 3.17, 95 per cent c.i.: 1.82–5.52, $P < 0.0001$, *Fig. 3a*) and DFS rate (HR = 2.95, 95 per cent c.i.: 1.73–5.03, $P < 0.0001$, *Fig. 3d*) among CRC patients. This significant association was also observed in the PSM population (*Fig. 3b,c*) and weighted cohort (*Fig. 3e,f*).

A univariate Cox model was used to investigate the impact of obstruction on survival outcomes (OS and DFS) in the entire

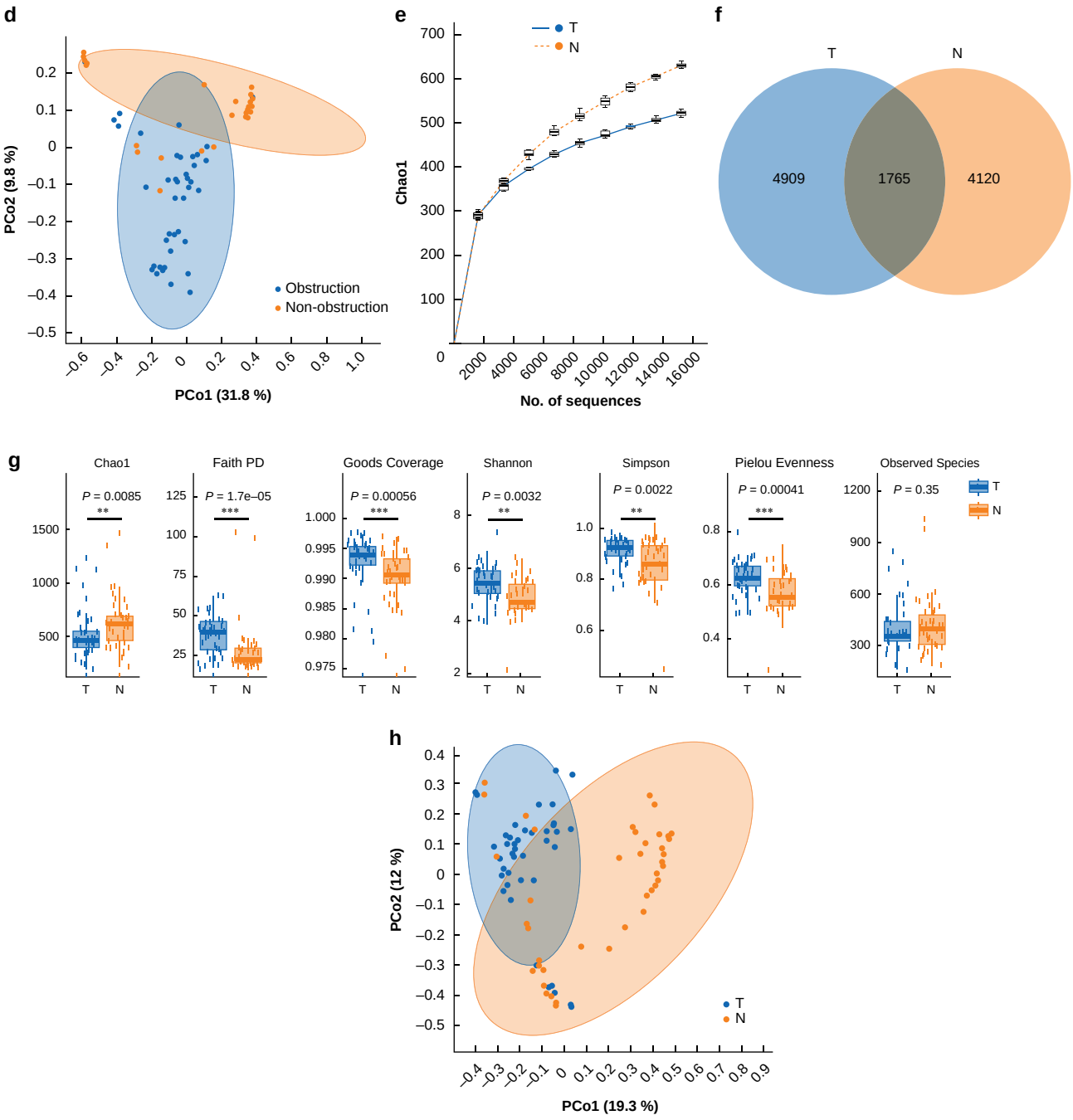

Fig. 4 Continued

population, PSM population and weighted cohort. As shown in *Table S1*, obstruction was a risk factor for poor survival outcomes in CRC patients. A sensitivity analysis was also conducted to confirm the positive association between obstruction and prognosis. After adjusting for potential confounding factors in the three models, this association remained significant ($P < 0.05$).

Composition of microbiota between obstruction and non-obstruction groups

A rarefaction curve was used to evaluate the adequacy of the sample size for the analysis. As depicted in *Fig. 4a,e*, the plateauing of the curves indicated that the sequencing depth of all samples was almost saturated, suggesting that the number of 16S rRNA sequencing reads was sufficient. A total of 15 651 OTUs were identified in our analysis, including 6674 in the obstruction group tumour tissues, 3204 in the non-obstruction group tumour tissues and 1238 OTUs in both groups (*Fig. 4b*). This indicated that there were significantly more OTUs in the obstruction group than in the non-obstruction group. The Venn diagram revealed that only 1765 of the 10 794 OTUs were unique to the obstruction tumour tissues compared to the non-tumour tissues (*Fig. 4f*).

Increased gut microbial alpha diversity in the obstruction colorectal cancer group

To assess microbial community richness and diversity within the samples, alpha diversity indices, including Chao1, Shannon and

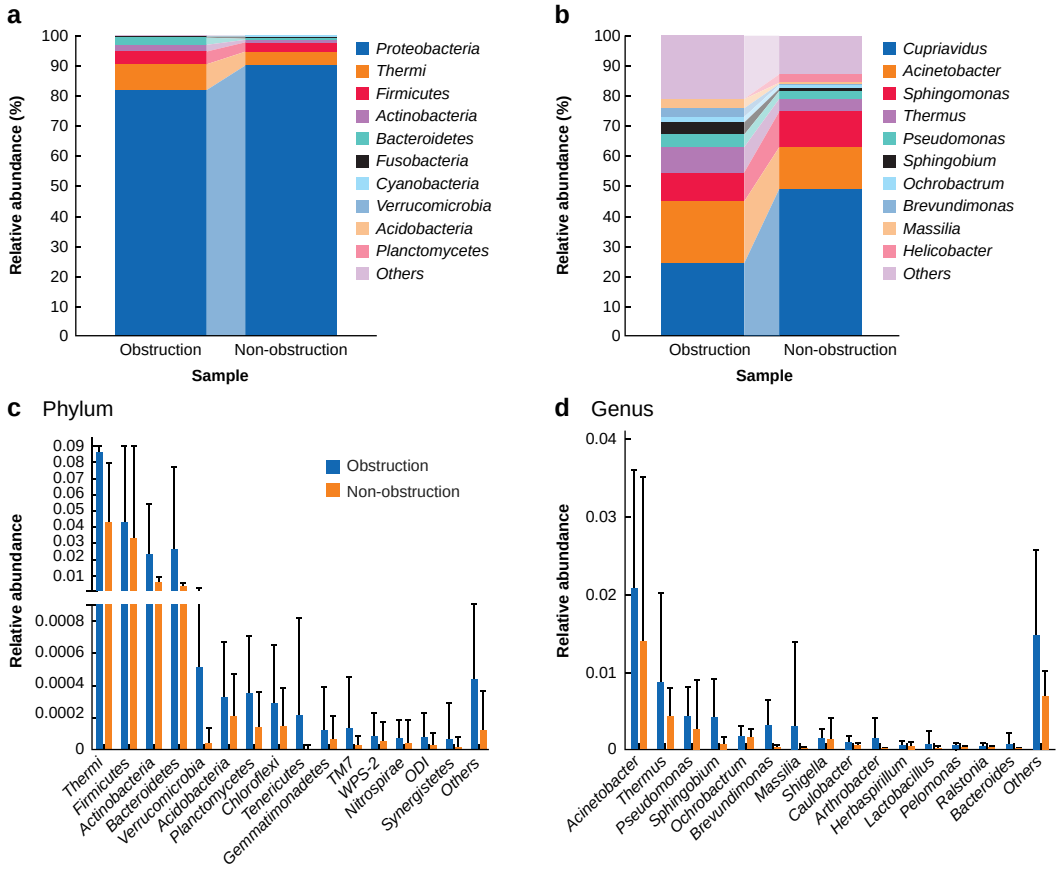

Fig. 5 Profiles of the microbial composition and differential communities at the phylum and genus levels (continued on next page)

Compositions of gut microbial taxonomic at the phylum (**a**) and genus (**b**) levels. The enriched microbial communities in obstruction tumour tissues versus non-obstruction, at the phylum level (**c**) and genus level (**d**). Upregulated and downregulated microbial taxonomy between obstruction and non-obstruction groups at the phylum (**e**) and genus (**f**) levels. Linear discriminant analysis effect size analysis revealed that there was remarkable difference in species diversity between the two groups (**g**). LDA, linear discriminant analysis; TM7, Saccharibacteria; OD1, Parcubacteria.

Simpson, were assessed. There were significant differences in ACE, Chao1, Shannon and Simpson indices between obstruction and non-obstruction tumour tissues ($P < 0.001$ for all indices, *Fig. 4c*). Additionally, significant differences were observed in ACE, Chao1 and Shannon indices between tumour and non-tumour tissues in the obstruction group ($P < 0.001$ for all indices, *Fig. 4g*). The Simpson index suggested that obstruction colorectal tumour samples had a larger variation in alpha diversity values than non-obstruction CRC samples.

To investigate the association between microbial alpha diversity and specific microflora, a correlation analysis was performed (*Table S2*). In the obstruction tumour tissues, the abundance of Proteobacteria showed an inverse correlation with alpha diversity, while the enrichment of Bacteroidetes and Firmicutes showed a positive correlation with alpha diversity (both $P < 0.05$). Beta diversity was assessed using PERMANOVA and the results indicated a significant difference in microbial composition between obstruction and non-obstruction CRC samples ($P = 0.001$, *Fig. 4d*). Additionally, significant differences were observed in both unweighted and weighted UniFrac distances between tumour and non-tumour tissues in the obstruction group ($P = 0.001$, *Fig. 4h*).

Microbial community composition in obstruction and non-obstruction colorectal cancer

As demonstrated in *Fig. 5a*, the most abundant phyla in both groups were Proteobacteria, Thermi, Firmicutes, Actinobacteria, Bacteroidetes, Fusobacteria, Cyanobacteria, Acidobacteria and Planctomycetes. At the genus level (*Fig. 5b*), the most abundant bacteria in both groups were *Cupriavidus*, *Acinetobacter*, *Sphingomonas*, *Thermus*, *Pseudomonas*, *Sphingobium*, *Ochrobactrum*, *Bacteroides*, *Massilia* and *Helicobacter*. However, there were differences in microbial community composition between the two groups. The Metastats comparison revealed that the abundance of Bacteroidetes was significantly higher in the obstruction group compared to the non-obstruction group (*Fig. 5c,d*).

There were differences in microbial taxa between the two groups at both the phylum and genus levels. For example, Firmicutes, Thermi, WPS-2, TM7, Actinobacteria, Planctomycetes, Synergistetes, OD1, Nitrospirae, Chloroflexi, Bacteroidetes, Verrucomicrobia, Tenericutes, Acidobacteria and Gemmatimonadetes were enriched in patients in the obstruction group, while Armatimonadetes, Deferribacteres, Fusobacteria, Proteobacteria and Cyanobacteria were enriched in the non-obstruction group (*Fig. 5e*). Similarly, at the genus level, *Ochrobactrum*, *Bacteroides*, *Thermus*, *Shigella*, *Flavobacterium*, *Sphingobium*, *Massilia*, *Brevundimonas*, *Arthrobacter*, *Herbaspirillum*, *Pelomonas*, *Acinetobacter*, *Lactobacillus*, *Caulobacter*, and *Ralstonia* were upregulated in the obstruction group, while *Sphingomonas*, *Clostridium*, *Cupriavidus*, *Helicobacter* and *Leptotrichia* were upregulated in the non-obstruction group (*Fig. 5f*). Linear discriminant analysis effect size (LEfSe) analysis further revealed a significant difference in species diversity between the two groups

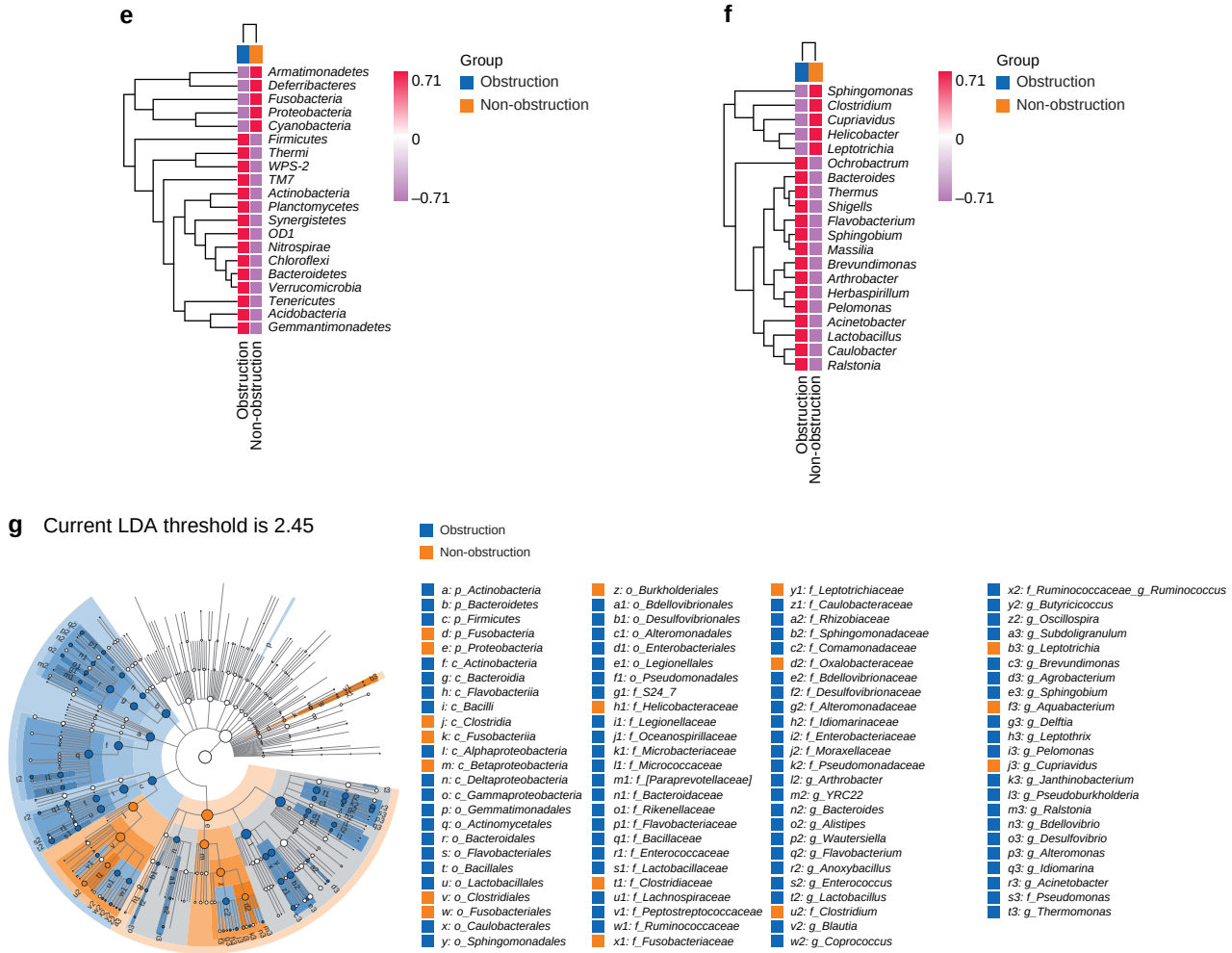

Fig. 5 Continued

(*Fig. 5g*). When the linear discriminant analysis (LDA) threshold was set at 2.45, a total of 98 species were identified in the obstruction and non-obstruction groups, with 81 species enriched in the obstruction group and 17 species enriched in the non-obstruction group.

Validation with frozen colorectal cancer tissues

The results were validated with frozen samples from 36 patients with newly diagnosed CRC and analysed with 16S rRNA sequencing. The rarefaction curve is shown in *Fig. 6a*. A total of 5459 OTUs were identified in our analysis, including 4283 in the obstruction group, 1172 in the non-obstruction group and 596 OTUs in both groups (*Fig. S3b*), indicating that OTUs were higher in the obstruction group. The most abundant phyla within the obstruction group and non-obstruction group at the phylum level (*Fig. 6c*) are Proteobacteria, Firmicutes, Thermi and Bacteroidetes. At the genus level, the most abundant bacteria within the two groups were *Cupriavidus*, *Acinetobacter* and *Sphingomonas* (*Fig. 6d*). The abundance within the obstruction group and non-obstruction group both at the phylum and genus levels based on frozen tissues was similar to the abundance based on paraffin tissue samples. At the phylum level, Bacteroidetes, Actinobacteria and Nitrospirae were upregulated in the obstruction group, while Proteobacteria and Fusobacteria were upregulated in the non-obstruction group (*Fig. 6e*); at the genus level, *Ochrobactrum*, *Bacteroides* and *Arthrobacter* were enriched in the obstruction group (*Fig. 6f*). Alpha and beta diversity was also different between the obstruction group and the non-obstruction group based on fresh tissues (*Fig. 7a,b*). Results of LEfSe analysis showed that there was a significant difference in species diversity between the two groups based on frozen tissues (*Fig. 7c*).

Comparison of immunofluorescence and the immune cells in the two groups

Invasive *Bacteroides* was prevalent in the obstruction group but rare in the non-obstruction group. *Bacteroides* positivity and high abundance were significantly more prevalent in the obstruction compared to that in the non-obstruction group (*Fig. 8*). The density of CD4+ and CD20+ T cells regarding positive cells in the IM seemed to be lower in the obstruction group than that in the non-obstruction group. As for CD8+ and CD68+ T cells, the positive cells in the IM seemed to be higher in the obstruction group than that in the non-obstruction group (*Fig. S1*). The insignificant difference of immune cells between the two groups is more likely due to the small sample size of CRC patients.

Association of Bacteroidetes status with clinical characteristics and outcomes

There was no significant association between Bacteroidetes status and clinicopathological characteristics of the 84 CRC patients (all $P > 0.05$, *Table S3*). Patients with high Bacteroidetes in obstruction CRC tissues had a shorter DFS time than

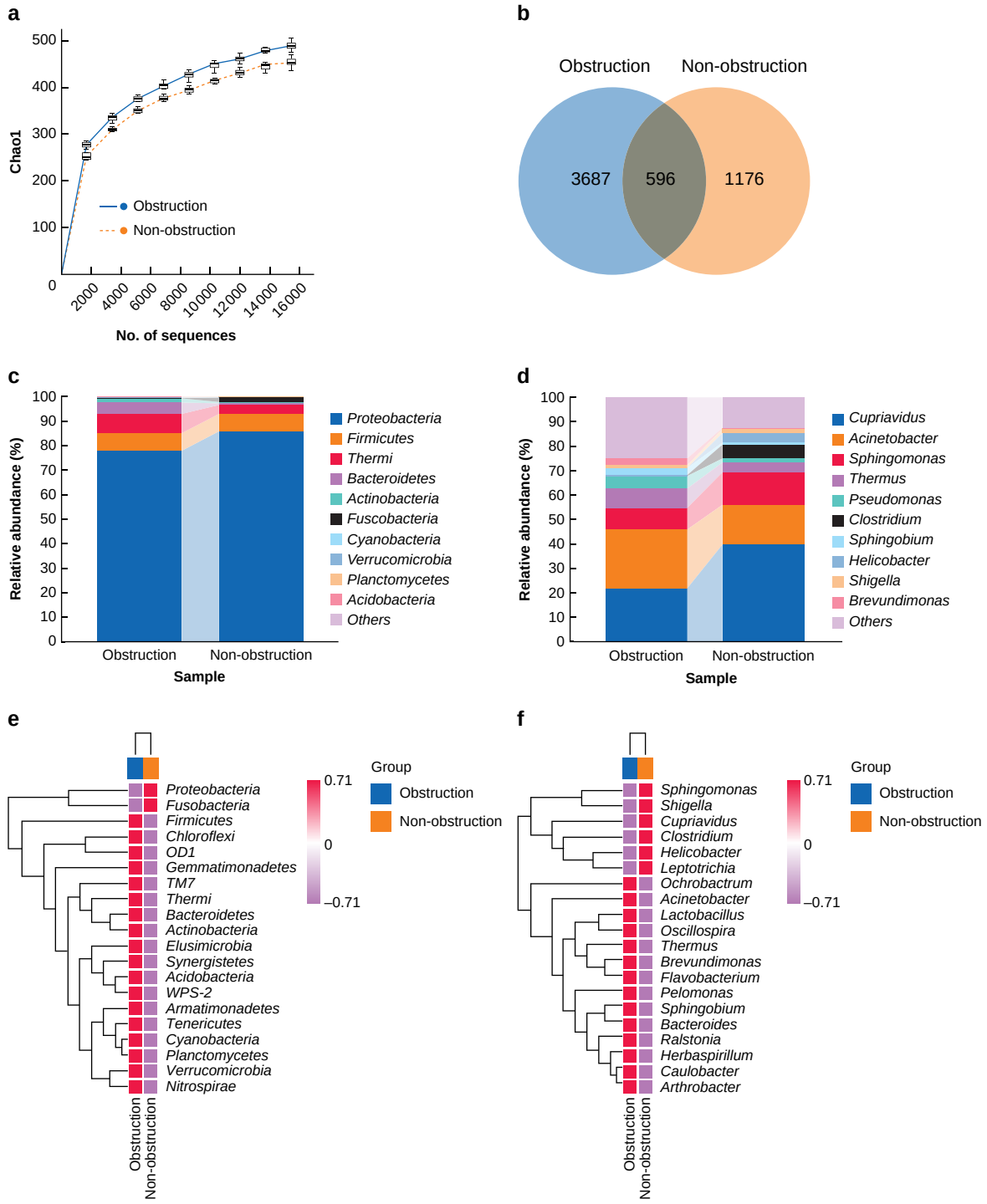

Fig. 6 Rarefaction curve and gut microbiota composition between obstruction and non-obstruction group

a. Rarefaction curve. **b**. The Venn plots reveal the unique and common taxa between obstruction and non-obstruction groups. Compositions of gut microbial taxonomic at the phylum (**c**) and genus (**d**) levels. Upregulated and downregulated microbial taxonomic between obstruction and non-obstruction groups at the phylum (**e**) and genus (**f**) levels.

Bacteroidetes-negative patients ($P = 0.037$, *Fig. 9a*), while the trend was not significant in the non-obstruction group CRC patients ($P = 0.001$, *Fig. 9b*). Deferribacteres-high CRC patients had a shorter DFS time than Deferribacteres-negative patients ($P < 0.001$, *Fig. 9c*). Stratification analyses of the association of Bacteroidetes status with OS time in 84 CRC showed that high Bacteroidetes in obstruction CRC tissues was associated with significantly shorter survival time ($P = 0.037$, *Fig. 9d*) than Bacteroidetes-negative populations, while the trend was not significant in the non-obstruction group CRC patients ($P = 0.835$, *Fig. 9e*). Similarly, Gemmatimonadetes-negative CRC patients had a shorter OS time than Deferribacteres-high patients, and this difference was statistically significant ($P < 0.001$, *Fig. 9f*). *Bacteroides* was the only bacterium that showed a significant association with both DFS and OS in obstruction patients. There was no association of other enriched microbial communities in obstruction tumour tissues with recurrence and survival time ($P > 0.05$, *Table 2*, and *Table S4*).

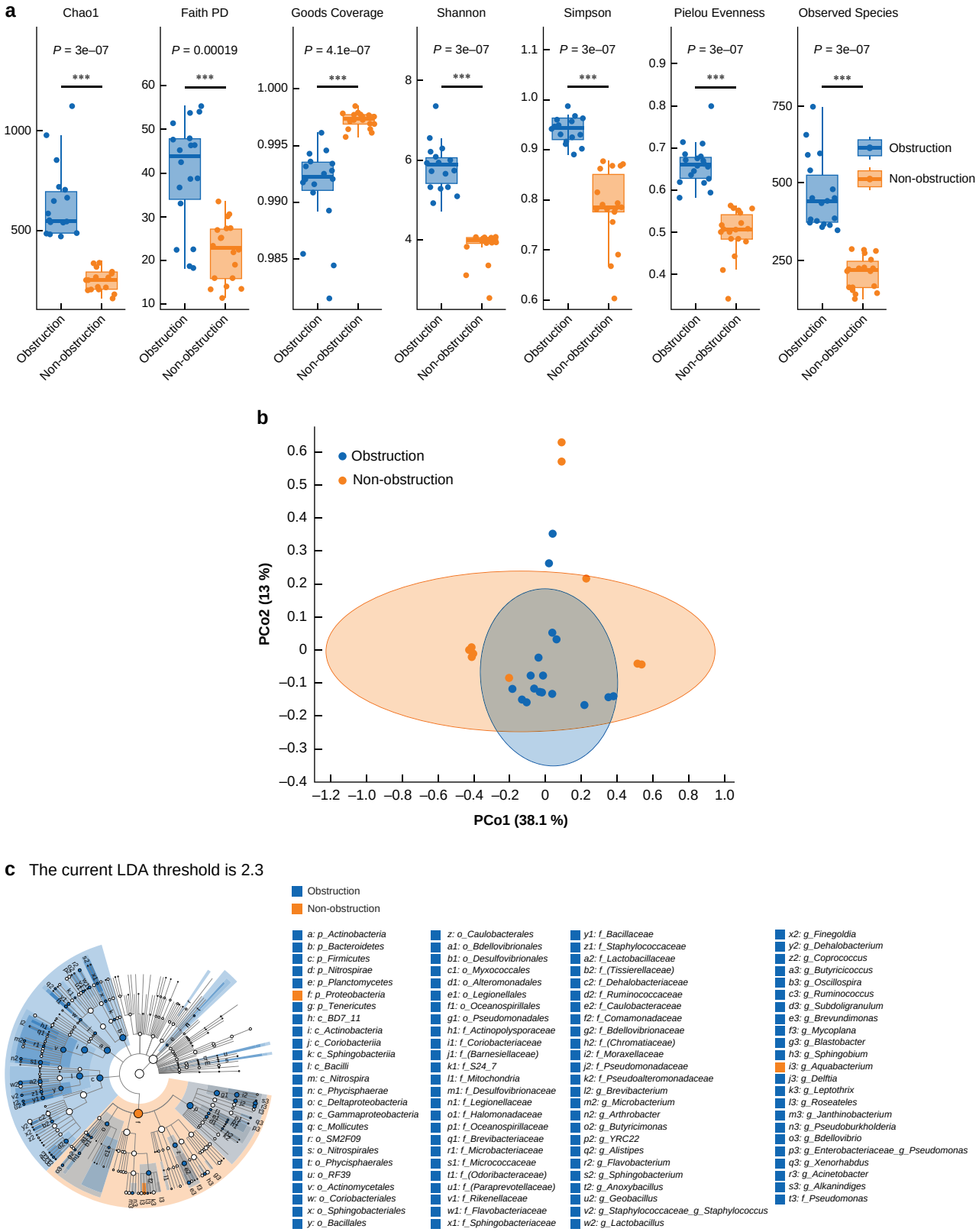

Fig. 7 Diversity of microbiota and Linear discriminant analysis effect size (LEfSe) analyses of the obstruction group and the non-obstruction group

a. Alpha diversity reveals that species richness was different between the two groups. **b**. Beta diversity analysis revealed by principal coordinate analysis. **c**. LEfSe analysis revealed that there was remarkable difference in species diversity between the two groups. LDA, linear discriminant analysis.

Correlation pathway analysis

The 16S rRNA sequencing results of CRC tissues were subjected to Kyoto Encyclopedia of Genes and Genomes (KEGG) and KEGG Orthology (KO) analyses to compare the functional pathways between obstruction and non-obstruction groups. The relative abundances of the top 5 biosynthesis pathways were identified,

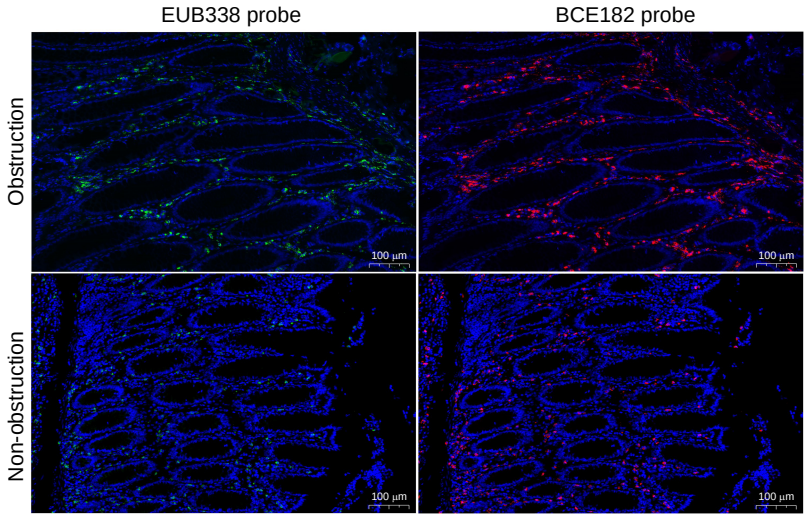

Fig. 8 Representative images of fluorescence *in situ* hybridization detecting invasive *Bacteroides* in obstruction and non-obstruction groups

Bacteroides positivity and high abundance was significantly more prevalent in the obstruction group compared to that in non-obstruction group. FISH using a Cy3-conjugated 'universal bacterial' 16S rRNA-directed oligonucleotide probe (EUB338, red); and FITC-conjugated *Bacteroides* 16S rRNA-directed oligonucleotide probe (BCE182, green) demonstrates the presence of bacteria and *Bacteroides* within the colonic mucosa of colorectal tumour samples. Epithelial cell nuclei were stained with DAPI. 100 × magnification.

including co-factor, prosthetic group, electron carrier, vitamin biosynthesis, amino acid biosynthesis, nucleoside and nucleotide biosynthesis, fatty acid and lipid biosynthesis and carbohydrate biosynthesis, as shown in *Fig. S2*. KO analysis identified eight significant metabolic pathways that differed between the obstruction and non-obstruction groups, as detailed in *Table S5* and illustrated in *Fig. S3*.

Discussion

The gut microbiota has been implicated in the tumorigenesis of CRC. Stools and biopsies from CRC patients have been reported to be rich in a variety of bacteria, which play a key role in shaping the inflammatory environment and can promote CRC tumour growth and metastasis[23–25]. In patients with intestinal obstruction, the intestinal mucosal barrier function is disrupted, which leads to dysbiosis. This disruption enables bacteria and toxins to enter the bloodstream through the intestinal mucosal barrier, exacerbating the inflammatory response of patients[26]. CRC complicated with intestinal obstruction has two defining features: tumour biology and intestinal obstruction. It is of great clinical significance to investigate the changes in gut microbiota that occur during the occurrence and development of CRC complicated with intestinal obstruction and any potential impact on prognosis.

In this study, the prognostic significance of obstruction for CRC patients with different survival risks was explored. Patients with obstruction due to CRC had worse OS and DFS. The composition of the gut microbiota between obstruction and non-obstruction patients using 16S rRNA gene high-throughput sequencing was compared. There were significant differences in the diversity and composition of the intestinal microbiome between obstruction and non-obstruction groups, suggesting that obstruction increases the diversity of gut microbiota in CRC patients. The results suggested that Bacteroidetes is an adverse prognostic factor, especially in obstruction patients.

It has been reported that compared to the non-obstruction CRC patients, those with obstruction are typically at an advanced stage, have a lower degree of postoperative tumour differentiation and have a higher likelihood of metastasis. Long-term prognosis is poor, with 5-year survival rates ranging between 31 and 42 per cent[27]. There are conflicting reports regarding the impact of intestinal obstruction on the prognosis of CRC patients. Some suggest that although intestinal obstruction is an emergency condition, it does not affect the long-term survival of patients[28]. In general, the survival time of CRC patients with obstruction is shorter than that of non-obstruction patients. However, heterogeneity in study design, stage, definitions, degree of obstruction, treatment strategies and adjuvant chemotherapy can make comparisons challenging[28,29]. This study focused on the clinical and long-term prognostic significance of obstruction among CRC patients and found that obstruction was associated with less-favourable survival outcomes in both the entire cohort and the PSM cohort.

Riquelme *et al.* reported that the tumour microbial composition of pancreatic cancer patients with different survival times was significantly different. They found that higher α diversity of tumour tissue was associated with longer survival time[30]. Another study utilized microbial-recognition chips to examine bacterial species in the upper gastrointestinal tissues and reported that reduced microbial abundance in the upper digestive tract was strongly associated with two cancer susceptibility states[31]. Although many of these studies had similar sample sizes between groups, the groups were still unbalanced in terms of baseline characteristics, which could introduce bias in their conclusions. As this study was a retrospective cohort analysis, propensity scores were calculated in the obstruction and non-obstruction groups to adjust for confounding variables. The gut microbiome is altered by many factors, including ethnicity, age, sex and TNM stage. By using a 1:1 PSM analysis to balance the obstruction and non-obstruction groups, intestinal obstruction was strongly associated with poor survival outcomes in CRC patients undergoing surgical resection. After PSM, gut microbiota composition differed significantly between the obstruction and non-obstruction groups, and alpha diversity in tumour tissues of the two groups of patients was closely associated with specific microbiota.

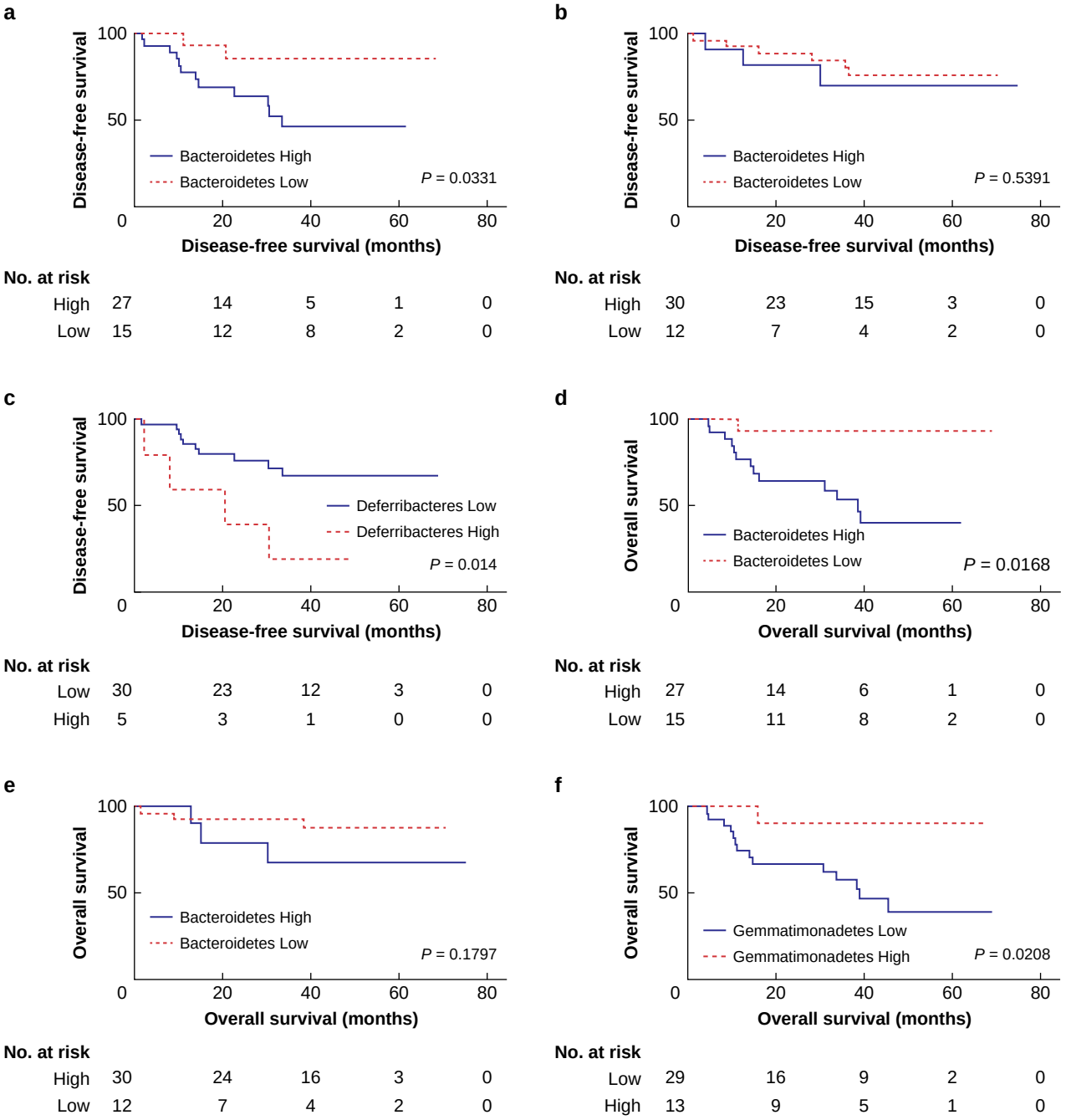

Fig. 9 Association of microbiota status with prognosis

The Kaplan–Meier curves for disease-free survival according to the relative status of Bacteroidetes in colorectal cancer (CRC) obstruction tumour tissues (**a**); the relative status of Bacteroidetes in non-obstruction tumour tissues (**b**); the relative status of Deferribacteres in obstruction tumour tissues (**c**); the Kaplan–Meier curves for overall survival according to the relative status of Bacteroidetes in obstruction CRC patients (**d**); the relative status of Bacteroidetes in non-obstruction tumour tissues (**e**); the relative status of Gemmatimonadetes in obstruction tumour tissues (**f**); cases with Bacteroidetes were categorized as high or low/negative using the medium value as cut-off point. *P* was calculated by the log-rank test.

In stage III and IV breast cancer, Gemmatimonadetes, Tenericutes and *Bosea* are the dominant microbiota, and have been shown to impact the patient prognosis[32]. In this study, the survival time of Deferribacteres-high CRC patients was significantly shorter than that of Deferribacteres-negative patients. A study by Pan *et al.* reported that reducing the relative abundance of Firmicutes and Deferribacteres led to a decrease in the number of intestinal CRC lesions in mice, suggesting a therapeutic role for this approach. Moreover, *Lactobacillus* was found to have a poor prognosis for CRC[33]. This study supports that Deferribacteres may be a negative prognostic factor in CRC, as Deferribacteres-high patients had a significantly shorter survival time compared to Deferribacteres-negative patients ($P < 0.05$).

Bacteroides is strongly associated with chronic intestinal inflammation, and increased numbers of *Bacteroides* have been detected in the intestines of patients with CRC[34]. Flemer *et al.* reported that Bacteroidetes, especially Cluster 2, showed increased abundance in CRC mucosal-associated lymphoid tissue, and some members of this phylum are responsible for

Table 2 Association of microbial communities with overall survival of obstruction and non-obstruction colorectal cancer

Microbial communities	Obstruction OS (months)			Non-obstruction OS (months)		
	High	Low/negative	P	High	Low/negative	P
Proteobacteria	47.8	48.7	0.931	–	–	0.430
Thermi	45.4	49.9	0.633	65.9	63.4	0.543
Firmicutes	44.0	48.8	0.917	–	–	0.526
Actinobacteria	47.4	44.2	0.953	–	65.0	–
Chloroflexi	46.5	47.8	0.693	–	65.0	–
Fusobacteria	–	48.7	–	–	–	0.660
Cyanobacteria	53.1	44.0	0.206	61.1	60.9	0.313
Verrucomicrobia	42.4	49.0	0.870	–	–	0.660
Acidobacteria	53.3	44.3	0.332	62.8	63.2	0.719
Planctomy	45.1	51.5	0.523	61.2	65.6	0.749
Bacteroidetes	37.0	64.5	0.008	57.0	63.6	0.180
Tenericutes	45.0	48.2	0.922	–	65.0	–
Gemmatimonadetes	62.1	42.8	0.032	69.0	60.0	0.659
TM7	43.1	51.5	0.350	65.6	61.8	0.973
Armatimonadetes	47.8	44.9	0.831	46.5	66.1	0.574
WPS2	54.9	42.6	0.438	50.9	68.5	0.063
Nitrospirae	57.4	39.1	0.072	–	–	0.311
OD1	54.2	42.5	0.436	–	–	0.389
Synergistetes	–	–	0.116	–	–	0.336
Deferribacteres	26.2	51.0	0.069	–	–	0.305

Level of microflora (high, low) is based on the average abundance of microbiota in tumour tissues. OS, overall survival; TM7, Saccharibacteria; OD1, Parcubacteria.

the degradation of host glycans and mucous layers, which they use as a nutritional and energy source, leading to disturbance of epithelial barriers. This can lead to gut barrier disruption, damaging the mucus layer and the epithelium and eventually inducing immune responses that may result in chronic inflammation and poor prognosis[35–37]. This study demonstrated that *Bacteroides* was enriched in the obstruction group at the genus level, indicating that *Bacteroides* plays an inflammatory role in the occurrence and progression of CRC complicated with intestinal obstruction. At the phylum level, Bacteroidetes was also upregulated in the obstruction group, further demonstrating their impact on the prognosis of patients. This is consistent with the findings of previous studies. There was high expression of Bacteroidetes in obstruction patients and this was significantly associated with poor DFS and OS. Bacteroidetes was the only microbiota that was significant for both DFS and OS in obstruction patients. This may be because Bacteroidetes act on tumour cells for a long time, affecting the occurrence and development of tumours and the prognosis of patients. However, the mechanism of action remains unclear, and further detailed exploration is necessary in the future.

There are some limitations to this study. First, this study was retrospective in nature and only paraffin tissue samples were available for analysis, which may have influenced the diversity of gut microbiota. Although the reliability was validated by using 16S rRNA sequencing of frozen CRC tissues, the sample size of newly collected tissues was limited due to time constraints. Second, other microbiological samples, such as saliva and stool samples, were not collected from patients to systematically compare the consistency of results. Lastly, this was a single-centre CRC database, and the retrospective analysis method may have some bias, and the level of evidence is not as strong as that of prospective studies. Despite these limitations, this study is the first to report differences in gut microbiota between CRC patients with and without obstruction and to identify the categories of differential bacteria and their impact on patient outcomes. This is of great significance for the prognostic study of CRC patients with intestinal obstruction and provides important new reference data for other researchers. In the future, it will be necessary to design prospective studies with larger sample sizes, collecting fresh tissue and faecal microbiota samples from patients to verify the reliability of the results.

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Y.C., F.S., and M.J. contributed equally to this study.

Disclosure

The authors declare that they have no conflict of interest.

Supplementary material

Supplementary material is available at *BJS Open* online.

Data availability

The data sets presented in this study can be found in online repositories. The names of the repositories and accession numbers can be found at: National Center for Biotechnology Information (NCBI), Sequence Read Archive (SRA), https://www.ncbi.nlm.nih.

gov/sra, SRR12019460-SRR12020117 and NCBI BioSample, https://www.ncbi.nlm.nih.gov/biosample/, SAMN30428202 -SAMN30428209.

Author contributions

Yinghao Cao (Formal analysis, Writing—original draft), Fumei Shang (Data curation, Methodology, Software, Writing—original draft), Min Jin (Data curation, Methodology), Shenghe Deng (Data curation, Methodology), Junnan Gu (Methodology), Fuwei Mao (Data curation), Le Qin (Data curation, Investigation), Ju Wang (Data curation, Software), Yifan Xue (Data curation, Software), Zhenxing Jiang (Data curation, Software), Denglong Cheng (Data curation), Li Liu (Project administration, Software, Validation), Xiu Nie (Conceptualization, Project administration, Supervision), Tao Liu (Conceptualization, Project administration), Hongli Liu (Funding acquisition, Resources, Visualization, Writing—review & editing), and Kailin Cai (Funding acquisition, Resources, Writing—review & editing)

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