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Observational Study

Longitudinal changes in the gut microbiota of Vietnamese patients with colorectal cancer undergoing surgery and chemotherapy

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Abstract

BACKGROUND

Colorectal cancer (CRC) is one of the leading common cancers worldwide. Emerging evidence indicates that the gut microbiota influences tumor progression and response to anti-CRC therapies. Yet, longitudinal studies tracking microbiota changes during the treatment period are rare, and none has been conducted in Vietnam.

AIM

To describe gut bacterial diversity and composition changes during CRC treatment, and their association with the response to treatment.

METHODS

Clinical data and fecal samples were obtained from 31 patients with CRC at diagnosis, after surgery, and after chemotherapy completion. After surgery, 13 patients received single-agent therapy [5-fluorouracil (5-FU)] and 18 received combination therapy (≥ 2 drugs, including 5-FU and oxaliplatin). Gut microbial diversity and community composition were characterized using 16S rRNA short-amplicon sequencing (V3-V4) of fecal genomic DNA, followed by downstream analysis with the QIIME2 pipeline.

RESULTS

In patients with CRC, the fecal microbiota alpha and beta diversity (unweighted UniFrac distance) and taxonomic composition changed over time during the treatment period. Alpha diversity decreased after surgery and after chemotherapy compared with baseline (diagnosis), and this was accompanied by the loss of several bacterial families and genera. Several pathogenic taxa, previously identified as overrepresented in patients with CRC relative to healthy individuals, strongly decreased after surgery and after chemotherapy, such as *Parvimonas*, *Peptostreptococcus*, *Porphyromonas*, *Desulfovibrio*, *Prevotella*, and *Turicibacter*. After chemotherapy, short-chain fatty acid-producing bacteria also were reduced, particularly within the genera NK4A214, UCG-005, and UCG-002 (Oscillospiraceae family), as well as the *Eubacterium ruminantium* and *Ruminococcus gauvreauii* species. Additionally, several taxa showed differences in function of the chemotherapy regimen and treatment response, which did not remain significant after multiple testing correction.

CONCLUSION

Following surgery and chemotherapy, the gut microbiota profile changed in patients with CRC, with loss of both disease-associated and beneficial bacteria. These results emphasize the potential of microbiota-targeted strategies for improving therapeutic outcomes and quality of life.

Key Words: Colorectal cancer; Chemotherapy; Longitudinal study; Microbiota profiling; Temporal dynamics; 5-fluorouracil; Oxaliplatin

Core Tip: Gut microbiota plays key roles in intestinal inflammation and tumor development, but also in shaping the anti-tumor immune responses. We longitudinally monitored the gut microbiota of patients with colorectal cancer (CRC) at diagnosis, after surgery and after chemotherapy. Alpha diversity declined and the overall microbial composition (unweighted UniFrac distance) changed at the three timepoints. Overall, CRC-associated pathogens and short-chain fatty acid-producing bacteria were decreased at post-treatment timepoints. Collectively, these results underscore the clinical potential of microbiota-targeted approaches to strengthen the gut barrier and enhance chemotherapy efficacy.

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INTRODUCTION

Colorectal cancer (CRC) ranks among the most frequently diagnosed cancers, including in Vietnam. Its incidence and mortality have declined in many high-income countries due to the widespread implementation of prevention strategies (*e.g.*, risk factor modification) and of screening and early detection programs as well as therapeutic advances. Conversely, rates continue to increase in many low- and middle-income countries[1,2]. Approximately two-thirds of CRC arise sporadically. Environmental and lifestyle factors play a predominant role, including obesity, physical inactivity, unhealthy dietary patterns (high intake of red and processed meat, excessive alcohol intake), cigarette smoking, and gut microbiome composition alterations[3].

The hypothesis linking the gut microbiota to CRC development was initially proposed in the early 1970s. In germ-free mouse models exposed to the carcinogen 1,2-dimethylhydrazine, CRC incidence was markedly reduced[4]. Besides its role in intestinal inflammation and tumorigenesis, the gut microbiota is also involved in regulating the antitumor immune responses[5]. Emerging evidence suggests that the gut microbiota may function as a predictive biomarker of immunotherapy outcomes, and its modulation could enhance treatment efficacy in CRC[6]. Studies on the gut microbiota's role in the tumor response to treatment represent a central research area and ongoing research focuses on different therapeutic modalities to facilitate clinical translation. Besides conventional chemotherapy and radiotherapy, recent advances have highlighted the synergistic interactions between gut microbiota and immune checkpoint inhibitors[7].

Yu *et al*[8] demonstrated that *Fusobacterium nucleatum* (*F. nucleatum*) is strongly enriched in CRC samples from patients resistant to chemotherapy. They suggested that in CRC, this bacterial species promotes chemoresistance by activating the autophagy pathway. The gut microbiota may also contribute to chemotherapy-induced toxicity. For instance, β-glucuronidases secreted by commensal bacteria, such as *Escherichia coli*, *Bacteroides spp*., and *Clostridium spp*., can reactivate the chemotherapeutic agent irinotecan, resulting in severe diarrhea in patients with CRC. In agreement, selective inhibition of these bacterial enzymes prevents irinotecan-induced diarrhea[9]. Fluoropyrimidines, namely 5-fluorouracil (5-FU) and capecitabine, are also cytotoxic agents frequently used in CRC. Specific bacterial species and their metabolic activities play a crucial role in regulating their metabolism and pharmacodynamics. Particularly, butyrate-producing bacteria, such as *Butyricicoccus*, *Lactobacillus*, *Clostridium butyricum*, and Ruminococcaceae family members, are responsible for butyrate production. This short-chain fatty acid (SCFA) mitigates 5-FU adverse effects and enhances its therapeutic efficacy[10]. Moreover, gut microbes can influence the therapeutic efficacy of 5-FU and other fluoropyrimidines by modulating nucleotide metabolism. Specifically, vitamin B6 and B9, produced by gut microbes, act as key regulators of bacterial folate metabolism, which directly influences 5-FU efficacy. Similarly, imbalances in bacterial deoxynucleotide pools enhance 5-FU efficacy by inducing the autophagy-mediated death of host cancer cells[11]. Given the critical role of the gut microbiota in modulating CRC treatment responses, we performed a longitudinal study of the gut microbiota composition and diversity in Vietnamese patients with CRC at key timepoints (diagnosis, after surgery and after chemotherapy). By characterizing the gut microbiota changes throughout the care pathway, we wanted to determine whether changes in gut microbial communities influence treatment efficacy. This information can contribute to define personalized therapeutic strategies.

MATERIALS AND METHODS

Patients' recruitment

Thirty-one patients with CRC were enrolled at diagnosis at the 108 Military Central Hospital, Hanoi, Vietnam, from 2022 to 2023.

Inclusion criteria: CRC resection surgery, adjuvant chemotherapy, and a follow-up period of ≥ 1 year, age between 50 years and 80 years, and body mass index (BMI) between 18.5 kg/m^2 and 30 kg/m^2.

Exclusion criteria: Complex medical conditions, mental health disorders, co-existing gastrointestinal diseases, recent antibiotic use (within 4 weeks before enrollment) or being on a restricted diet.

Fecal sample and clinical data collection

Fecal samples and clinical data were systematically obtained at three timepoints: (1) Before treatment (at diagnosis), at least 1 week after colonoscopy (T0); (2) At least 3 weeks after surgery to allow gut microbiota recovery, as patients underwent bowel cleansing before surgery and received a postoperative gastrointestinal diet and standardized perioperative antibiotics according to hospital protocols (T1); and (3) 4-6 weeks after the final chemotherapy dose, or at the transition to maintenance therapy in patients with metastatic disease (T2; Supplementary Figure 1). The sampling time points were selected based on prior observations of gut microbiota changes in CRC patients undergoing radical surgery and CAPEOX therapy[12] and were aligned with routine clinical follow-up schedules to ensure feasibility and patient compliance. Patients collected fecal samples in sterile plastic containers, at home or in hospital. All samples were stored at -80 °C before DNA extraction. The serum biochemical profiles, overall health status, clinical data and treatment type were retrieved from the patients' medical records. Demographic information (age, sex, BMI, family history of cancer) was acquired through interviews with the participants.

Genomic DNA extraction and 16S rRNA gene amplicon sequencing

Total DNA was extracted from 0.25 g of fecal material using the QIAamp® PowerFecal® DNA Kit (QIAGEN, Hilden, Germany) following the manufacturer's instruction. Before extraction, samples underwent mechanical homogenization using a TissueLyser II (QIAGEN, Hilden, Germany), with two cycles of 5 minutes at 25 Hz to maximize cell lysis. Following extraction, DNA was quantified with Qubit™ 1 × dsDNA HS Assay Kits. The 16S rRNA short-amplicon sequencing (V3-V4 regions) was carried out using the MiSeq sequencing system (Illumina, San Diego, CA, United States) as previously described[13].

Bioinformatics analysis for the sequencing data

The 250-bp paired-end Illumina reads were processed using Quantitative Insights in Microbial Ecology 2 (QIIME2) v.2024.5[14]. Both forward and reverse primer sequences were removed with cutadapt v.4.9, and trimmed reads were

denoised using DADA2 implemented in QIIME2. Taxonomic assignment of representative amplicon sequence variants (ASVs) from phylum to genus was performed using the QIIME2 feature-classifier (classify-sklearn) and the SILVA v138 full-length 16S rRNA reference database[15]. Unassigned sequences and those annotated as Eukaryota were excluded before phylogenetic tree construction with the align-to-tree-mafft-fasttree pipeline in the QIIME2 phylogeny module. Alpha and beta diversities were calculated using the QIIME2 core-metrics-phylogenetic workflow after rarefying the ASV table to the minimum sequencing depth observed across samples. As all samples exceeded the rarefaction threshold of 140511 reads, no sample was excluded from downstream analyses. Rarefaction was applied to ensure the comparability of alpha diversity metrics and UniFrac-based analyses in the QIIME2 framework.

Statistical analysis

Statistical analyses were performed in R v4.5.1[16]. Taxonomic tables were filtered to retain only taxa present in ≥ 50% of samples at each timepoint. The significance of alpha diversity indices and taxon relative abundances, stratified by clinical variables at different timepoints, was evaluated using the Mann-Whitney *U* test (n = 2 independent groups) or the Kruskal-Wallis test ($n \geq 3$ independent groups), followed by the Dunn's *post hoc* test for pairwise comparisons. Due to limited patient numbers per subgroup, all subgroup analyses stratified by clinical variables such as tumor location, treatment regimen, or therapeutic response were performed on an exploratory basis. Longitudinal changes in alpha diversity were assessed using the Friedman test, followed by the Nemenyi *post hoc* test for pairwise comparisons. Differential taxonomic abundance between timepoints was assessed using linear mixed-effects models implemented in LinDA [17], ANCOM-BC2[18] and MaAsLin 2[19], with patient included as covariate and default parameters. A pseudo-count of 0.5 was added to the absolute abundance count table before using the ANCOM-BC2 method to account for the absence of several taxa at specific timepoints. *P* values were adjusted for multiple testing using the Benjamini-Hochberg (BH) method. Beta diversity patterns were visualized using principal coordinates analysis, and statistical differences between groups were assessed using permutational multivariate analysis of variance (PERMANOVA) with 999 permutations. Spearman's rank correlations between ASV abundances and serum biochemical markers were calculated, and the results were visualized using ComplexHeatmap v.2.24.1.

RESULTS

Patients' clinical characteristics

A total of 31 patients with newly diagnosed CRC (stages II, III, and IV) who underwent resection surgery and chemotherapy were included (Table 1). The patients' mean age was 64 ± 8 years (range: 50-80 years). The most common clinical symptoms at diagnosis were abdominal pain and hematochezia. Two male patients experienced weight loss and were classified as underweight at diagnosis. Tumor size ranged from 2 cm to 8 cm (median size = 4.5 cm) and was 2-5 cm in 54.8% of patients. Left and right colon were the most common tumor sites [14 (45.2%) and 9 (29.0%) patients, respectively]. After surgery, 13 patients (41.9%) received 5-FU alone, and 18 patients (58.1%) received combination regimens (≥ 2 chemotherapy drugs). Capecitabine (25.8%) and mFOLFOX6 (35.5%) represented 61.3% of all treatment protocols. After chemotherapy completion, complete response was observed in 25 patients (80.6%), partial response in 3 (9.7%), and disease progression in 3 (9.7%). All patients survived for at least 12 months after diagnosis.

Most blood biochemical parameters were within the normal range at all timepoints. Similarly, hemoglobin concentration did not significantly change before and after chemotherapy. Carcinoembryonic antigen (CEA) concentration exceeded the threshold of 5 ng/mL in 4/31 patients at T0. This marker remained abnormal in two patients at T1 and T2, suggesting persistent tumor activity or residual disease. These two patients were among the three patients with partial response to treatment (Supplementary Table 1).

Loss of gut microbial diversity after chemotherapy

Alpha diversity (*i.e.*, observed ASVs and Faith's phylogenetic diversity) was lower at T1 and T2 than at T0 (Friedman test, $P < 0.05$). However, both the observed ASVs index ($P = 0.001$) and Faith's phylogenetic diversity ($P = 0.014$) were significantly different only between T0 and T2 (pairwise comparisons with the Nemenyi test). Conversely, the Pielou's evenness and Shannon's diversity indices did not differ across timepoints (Friedman test, $P > 0.05$; Figure 1A).

Beta diversity (unweighted UniFrac index) allowed clustering samples according to the timepoint (PERMANOVA: $R^2 = 0.086$; $P = 0.001$). T1 samples were between the T0 and T2 samples, but closer to the T2 samples, indicating a transitional state during treatment. Conversely, the weighted UniFrac index, which accounts for the relative abundance, was not different across timepoints (PERMANOVA: $R^2 = 0.026$; $P = 0.234$; Figure 1B).

Consistent with the unweighted UniFrac results, several bacterial taxa were no longer detected after surgery and after chemotherapy (Supplementary Table 2). Forty-three families (Supplementary Figure 2) did not change across timepoints (cluster a). Conversely, 18 families were detected in the gut microbiota of most patients at T0, but not always at T1 and T2 (cluster b). This might reflect reduced prevalence or abundance below the detection threshold rather than complete eradication. In cluster b, the presence/absence of 13/18 families changed significantly across timepoints (BH-adjusted $P < 0.05$, Cochran's *Q* test). Notably, the Cyclobacteriaceae, Hymenobacteraceae, Sphingobacteriaceae, and Alcaligenaceae families were present before treatment but not at the post-treatment timepoints (Supplementary Figure 2 and Supplementary Table 2). At the genus level, the *Eubacterium ruminantium* (*E. ruminantium*) group, *Solobacterium*, *Ruminococcus gauvreauii* (*R. gauvreauii*) group, *Peptostreptococcus*, *Parvimonas*, *Slackia*, *Prevotella*, *Desulfovibrio*, and *Oscillospiraceae NK4-A214* group were detected in most patients at T0, but were absent in several patients at T1 and T2 (BH-adjusted *P* values

Table 1 Clinical characteristics of patients with colorectal cancer, *n* (%)

Characteristic	Categories	Number of patients
Age at diagnosis (years): 64.3 ± 8 years	50-60	10 (32.3)
	60-70	10 (32.3)
	70-80	11 (35.5)
BMI	Normal weight	25 (80.6)
	Overweight	4 (12.9)
	Underweight	2 (6.5)
Sex	Female	11 (35.5)
	Male	20 (64.5)
Comorbidity	Type 2 diabetes	6 (19.4)
	Hypertension	7 (22.6)
Family history of CRC		3 (9.7)
Tumor size (cm)	2-5	17 (54.8)
	5-10	14 (45.2)
TNM stage	Stage II	9 (29)
	Stage III	18 (58.1)
	Stage IV	4 (12.9)
Tumor location	Left colon	14 (45.2)
	Right colon	9 (29)
	Transverse colon	2 (6.5)
	Rectum	6 (19.4)
Metastasis	Yes	6 (19.4)
	No	25 (80.6)
Treatment regimen	Single agent (5-FU)	13 (41.9)
	Capecitabine	8 (25.8)
	Ufur	4 (12.9)
	TS1	1 (3.2)
	Combination therapy (≥ 2 agents including 5-FU and oxaliplatin)	18 (58.1)
	mFOLFOX6	11 (35.5)
	XELOX	5 (16.1)
	B-mFOLFOX6	2 (6.5)
Therapy response	Complete response	25 (80.6)
	Partial response	3 (9.7)
	Disease progression	3 (9.7)

BMI: Body mass index; CRC: Colorectal cancer; 5-FU: 5-fluorouracil; TNM: Tumor, node and metastasis.

< 0.05, Cochran's *Q* test; Supplementary Table 2 and cluster a, Figure 1C). Similarly, the *Porphyromonas*, *Sphingomonas*, *Sphingobacterium*, *Salinimicrobium*, *Algoriphagus*, *Sporacetigenium*, *Pseudomonas*, *Lysobacter*, *Pontibacter*, *Chitinophagaceae UTBCD1*, and *Brevundimonas* genera were largely absent or undetectable in patients at T1 and T2 (Supplementary Table 2 and cluster b, Figure 1C). Conversely, *Megasphaera* and *Ignavibacteria SJA-28*, detected at T2, and the *Lachnospiraceae NK4A136* group, detected at T1, were absent at T0 (Supplementary Table 2 and cluster c, Figure 1C).

Gut microbiota diversity and clinical variables

The analysis of alpha diversity variations in function of the clinical variables did not highlight any difference relative to age, BMI, sex, type 2 diabetes, hypertension, tumor size, cancer stage, metastasis, or CEA level at T0 (Table 2). At T1,

Table 2 Alpha diversity of gut microbiota in function of the clinical variables at the three timepoints

	Pielou's evenness	Observed ASVs	Faith's phylogenetic	Shannon's entropy
Baseline (diagnosis; T0)				
Age groups (year): 50-59 (n = 10), 60-69 (n = 10) *vs* 70-79 (n = 11)	0.729	0.428	0.260	0.668
BMI categories: Normal weight (n = 25) *vs* overweight (n = 4)	0.562	0.591	0.482	0.521
Sex: Male (n = 11) *vs* female (n = 20)	0.113	0.901	0.919	0.261
Type 2 diabetes: Yes (n = 6) *vs* no (n = 25)	0.827	0.671	0.643	0.827
Blood pressure: Hypertension (n = 7) *vs* normal (n = 24)	0.982	0.508	0.563	0.800
Tumor size (cm): 2-5 (n = 17) *vs* 5-10 (n = 14)	0.922	0.843	0.984	0.891
TNM stage: Stage II (n = 9), stage III (n = 18) *vs* stage IV (n = 4)	0.908	0.982	0.814	0.892
Tumor location: Left colon (n = 14), right colon (n = 9) *vs* rectum (n = 6)	0.464	0.562	0.599	0.671
Metastasis: Yes (n = 6) *vs* no (n = 25)	0.291	0.635	0.679	0.339
CEA: Normal (n = 27) *vs* elevated (n = 4)	0.589	0.227	0.376	0.476
After surgery (T1)				
Age groups (year): 50-59 (n = 10), 60-69 (n = 10) *vs* 70-79 (n = 11)	0.608	0.902	0.705	0.851
BMI categories: Normal weight (n = 24) *vs* underweight (n = 5)	0.889	0.030[a]	0.044[a]	0.716
Sex: Male (n = 11) *vs* female (n = 20)	0.451	0.265	0.640	0.317
Type 2 diabetes: Yes (n = 6) *vs* no (n = 25)	0.053	0.635	0.643	0.105
Blood pressure: Hypertension (n = 7) *vs* normal (n = 24)	0.908	0.943	0.695	1.000
Tumor size (cm): 2-5 (n = 17) *vs* 5-10 (n = 14)	0.653	0.512	0.279	0.421
TNM stage: Stage II (n = 9), stage III (n = 18) *vs* stage IV (n = 4)	0.624	0.441	0.972	0.422
Tumor location: Left colon (n = 14), right colon (n = 9) *vs* rectum (n = 6)	0.050	0.008[a]	0.050	0.019[a]
After chemotherapy (T2)				
Age groups (year): 50-59 (n = 10), 60-69 (n = 10) *vs* 70-79 (n = 11)	0.118	0.083	0.316	0.108
BMI categories: Normal weight (n = 25) *vs* overweight (n = 4)	0.482	0.635	0.604	0.482
Sex: Male (n = 11) *vs* female (n = 20)	0.528	0.591	0.823	0.670
Type 2 diabetes: Yes (n = 6) *vs* no (n = 25)	0.643	0.960	0.751	0.715
Blood pressure: Hypertension (n = 7) *vs* normal (n = 24)	0.104	0.015[a]	0.234	0.054
Tumor size (cm): 2-5 (n = 17) *vs* 5-10 (n = 14)	0.356	0.439	0.570	0.316
TNM stage: Stage II (n = 9), stage III (n = 18) *vs* stage IV (n = 4)	0.373	0.138	0.439	0.314
Tumor location: Left colon (n = 14), right colon (n = 9) *vs* rectum (n = 6)	0.992	0.555	0.519	0.993

CEA: Normal (n = 24) *vs* elevated (n = 7)	0.094	0.026[a]	0.139	0.061
Treatment regimen: Single agent (n = 13) *vs* combination therapy (n = 18)	0.106	0.149	0.332	0.115
Therapy response: Responder (n = 28) *vs* non-responder (n = 3)	0.635	0.124	0.681	0.503

[a]$P < 0.05$.

P values were calculated with the Mann-Whitney *U* test (2 groups) and Kruskal-Wallis test (> 2 groups). Body mass index categories: Underweight (T0, T2) and overweight (T1) groups with ≤ 2 samples excluded due to insufficient sample size for statistical analysis. Tumor location: Transverse colon with 2 samples excluded. Carcinoembryonic antigen (CEA): Post-surgery (T1) CEA was not analyzed because concentration was elevated in only two patients. ASV: Amplicon sequence variant; BMI: Body mass index; TNM: Tumor, node and metastasis; CEA: Carcinoembryonic antigen.

microbial diversity was higher in patients with underweight than in those with normal weight: Median [interquartile range (IQR)] observed ASVs and Faith's phylogenetic diversity values = 391 (31.2) and 22.1 (3.3) in underweight patients and 330 (67.5) and 18.6 (2.8) in normal weight patients, respectively. Additionally, the observed ASVs and Shannon diversity indices differed by tumor location (Kruskal-Wallis test, $P < 0.05$), but not the Pielou's evenness and Faith's phylogenetic diversity indices ($P = 0.05$). The *post hoc* analysis indicated that observed ASVs was higher in samples from patients with left than right colon and rectum tumors, and Shannon diversity was higher in the right colon tumor subgroup (Dunn's test, $P < 0.05$; Supplementary Figure 3A). At T2, the median (IQR) Observed ASVs value was significantly higher in patients with hypertension than with normal blood pressure [347 (47) *vs* 298.5 (109)], and in patients with normal than with elevated CEA concentration [330 (64) *vs* 288 (45)]. Alpha diversity metrics were not different in function of the treatment regimen or response (Table 2).

The global composition of the gut microbiota (beta diversity) was not associated with clinical characteristics, except for the unweighted UniFrac distances with tumor location at T1 (PERMANOVA: $R^2 = 0.103$; $P = 0.019$), but not at T2 (Supplementary Figure 3B and Supplementary Table 3).

Gut microbiota composition changes during CRC treatment

Comparison of the relative abundance of bacterial taxa in each patient at the three timepoints highlighted fluctuations in bacterial composition during treatment (Supplementary Figure 4). Nevertheless, the dominant taxa remained largely stable over time. The Firmicutes, Bacteroidota, Proteobacteria, and Actinobacteriota phyla were consistently detected at all timepoints (Supplementary Figure 4A) as well the Lachnospiraceae, Ruminococcaceae, Enterobacteriaceae, Bacteroidaceae, and Peptostreptococcaceae families. For instance, Lachnospiraceae relative abundance (mean ± SD) was 42.04% ± 16.25% at T0, 40.51% ± 12.59% at T1, and 46.88% ± 17.59% at T2 (Supplementary Figure 4B). *Blautia* (Lachnospiraceae family) was the most dominant genus at all timepoints: 19.92% ± 11.10% at T0, 18.73% ± 9.58% at T1, and 22.19% ± 12.15% at T2. Other genera with lower abundances included *Escherichia-Shigella*, *Bacteroides*, *Faecalibacterium*, and *Clostridium sensu stricto 1* (*C. sensu stricto 1*; Supplementary Figure 4C).

Next, bacterial abundances at T1 and T2 were compared with the diagnosis timepoint (T0) using LinDA, MaAsLin 2 and ANCOM-BC2. The significant signals were largely consistent, particularly between LinDA and MaAsLin2. The abundance of the Nitrospirota and Chloroflexi phyla was higher at T2 than at T0, whereas that of *Fusobacteriota* was lower at T1 and T2 compared with T0 (Supplementary Figure 5 and Supplementary Tables 4-6). Moreover, 36 families exhibited substantial differences in abundance at the post-treatment timepoints compared with T0. *Ignavibacteria SJA-28*, Veillonellaceae, Streptococcaceae, and Bifidobacteriaceae were enriched exclusively at T2 (LinDA and MaAsLin2 or ANCOM-BC2). Conversely, 14 families (Sphingobacteriaceae, Cyclobacteriaceae, Sphingomonadaceae, Fusobacteriaceae, Alcaligenaceae, Porphyromonadaceae, Flavobacteriaceae, Chitinophagaceae, Gemellaceae, Hymenobacteraceae, Caulobacteraceae, Xanthomonadaceae, Peptostreptococcales-Tissierellales Family XI, and Pseudomonadaceae) were consistently depleted at T1 and T2 compared with T0 (at least two methods; Supplementary Figure 5 and Supplementary Tables 4-6). Moreover, 37 genera were differentially abundant at T1 or T2 compared with T0. The abundance of 5 genera (*Parasutterella*, *Megasphaera*, *Ignavibacteria SJA-28*, *Anaerotruncus*, and *Lachnospiraceae NK4A136* group) was significantly increased at T1 or T2 compared with T0, whereas that of the other 32 genera was decreased. The abundance of the *Parvimonas*, *Pseudomonas*, *Peptostreptococcus*, *Brevundimonas*, *Chitinophagaceae UTBCD1*, *Lysobacter*, *Pontibacter*, *Gemella*, *Porphyromonas*, *Sphingobacterium*, and *Fusobacterium* genera consistently decreased after treatment compared with T0 (Figure 2 and Supplementary Tables 4-6).

Exploratory analysis of potential association of the intestinal microbiota and clinical characteristics

As differences in both alpha and beta diversity were observed across tumor locations at T1, gut microbiota composition was compared in patients stratified by tumor location. The abundance of 12 (T1) and 7 (T2) genera was significantly different in function of the tumor location (uncorrected *P* values < 0.05; Supplementary Table 7). At T1, the abundance of *C. sensu stricto 1*, *Eubacterium coprostanoligenes* group, and *Oscillospiraceae UCG-005* was relatively higher in samples from patients with left colon tumors than with right colon and rectal tumors. Moreover, the *Eubacterium brachy* group and family XIII AD3011 group belonging to the Anaerovoracaceae family were enriched in patients with left colon tumors compared with rectal tumors. Conversely, the abundance of *Prevotella* 9 was lower in patients with right colon tumors compared with patients with left colon and rectal tumors. At T2, the abundance of the *Ruminococcus gnavus* group,

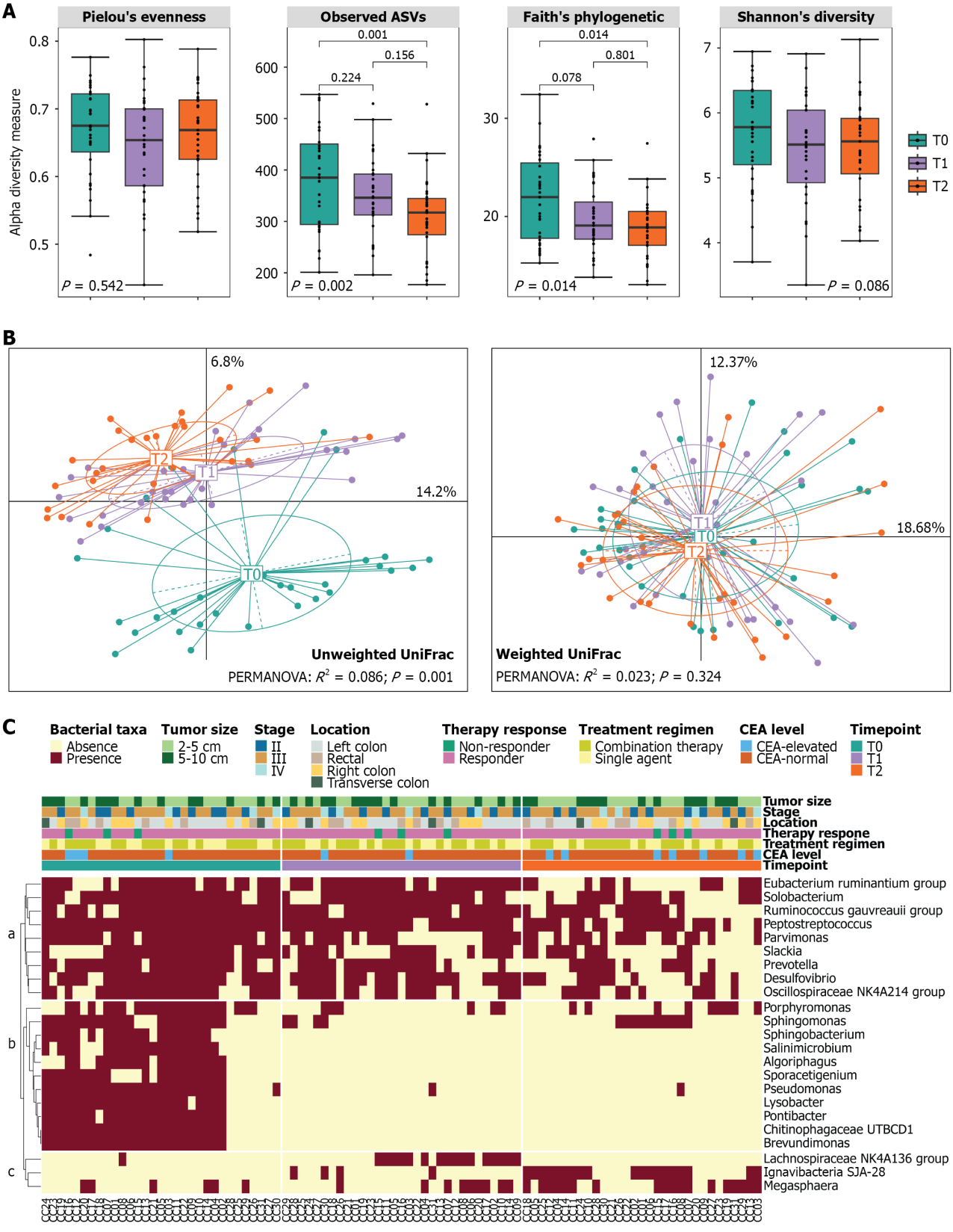

Figure 1 Microbial diversity of the gut microbiota in patients with colorectal cancer at diagnosis (T0), after surgery (T1), and after chemotherapy (T2). A: Boxplots showing the Pielou's evenness, observed amplicon sequence variants, Faith's phylogenetic diversity, and Shannon's diversity alpha diversity indices. *P* values were calculated with the Friedman test, and the Nemenyi test for pairwise comparisons when the Friedman test was significant; B: Principal coordinates analysis based on the unweighted and weighted UniFrac distances. Differences in beta diversity between timepoints were assessed using permutational multivariate analysis of variance; C: Heatmap showing the presence (red) and absence (light yellow) of gut microbiota genera. Only classified genera with significant differences across the three timepoints (Benjamini-Hochberg-adjusted *P* value < 0.05, Cochran's *Q* test) are displayed. Clustering of genera on the left side of the heatmap was performed using Euclidean distances and Ward's hierarchical algorithm, resulting in three major clusters (a, b, and c) based on their presence across

samples. Colored bars at the top of the heatmap represent tumor size, tumor, node and metastasis stage, location, therapy response, treatment regimen, carcinoembryonic antigen concentration, and timepoint.

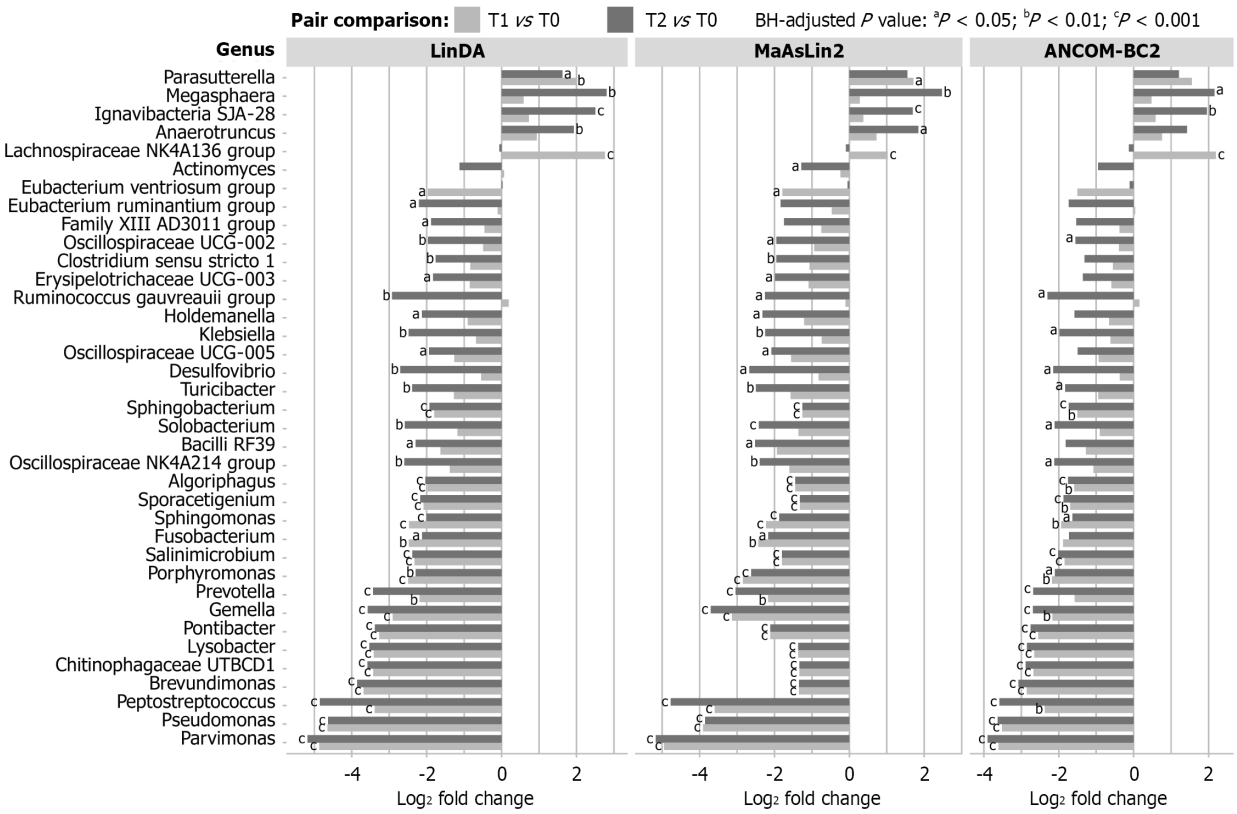

Figure 2 Temporal gut microbiota changes at the genus level during the treatment period. Differentially abundant bacterial genera at post-treatment timepoints relative to T0 (diagnosis) were identified using LinDA, MaAsLin2, and ANCOM-BC2. Differences between T1 (after surgery) and T0 and between T2 (after chemotherapy) and T0 are shown as $\log_2$ fold changes. Only classified genera with significant differences between timepoints are presented. [a]Adjusted $P < 0.05$; [b]Adjusted $P < 0.01$; [c]Adjusted $P < 0.001$.

Dialister, *Fusobacterium*, *Leuconostoc*, *Fonticella*, *Nitrospirota* 4-29-1, and unclassified *Xanthobacteraceae* genera differed in function of the tumor location. Notably, none of the differentially abundant taxa was shared between T1 and T2. However, all associations lost statistical significance after adjustment for multiple comparisons (Supplementary Figure 6 and Supplementary Table 7).

The next question was whether the intestinal microbiota at T2 was associated with the treatment regimen or therapy response. Due to the small number of patients per regimen, patients were grouped into single-agent therapy (5-FU) and combination therapy (≥ 2 chemotherapy drugs) for treatment-regimen comparison. At T2, the abundance of *Adlercreutzia*, *Lactonifactor*, *Ruminococcus*, *Klebsiella*, and unclassified Enterobacteriaceae was higher in the combination therapy than single-agent therapy subgroup, whereas *Butyricimonas* showed the opposite trend (Mann-Whitney *U* test, uncorrected $P < 0.05$; Figure 3). Seven genera (*Bacteroidetes vadinHA17*, *Ktedonobacterales JG30-KF-AS9*, *Lactococcus*, *Subdoligranulum*, and *Sphingomonas)* were enriched in non-responders ($n = 3$), whereas *Eubacterium* and *Oscillospiraceae UCG-005* were more abundant in responders ($n = 28$; Mann-Whitney *U* test, uncorrected $P < 0.05$; Supplementary Figure 7). However, the sample size imbalance between responders and non-responders may have led to false-positive associations. All these differences in function of the treatment regimen or response were not significant after multiple testing corrections (Supplementary Table 7). Nevertheless, the initial trends suggest that clinical characteristics, such as tumor location, treatment regimen, and therapy response, may influence the intestinal microbiota profile after treatment.

Serum biochemical markers and gut microbiota abundance

The analysis of the correlations between ASV abundances and serum biochemical markers found weak but statistically significant associations (Spearman's $\rho < 0.5$, adjusted *P* values < 0.05) of 36 ASVs with glucose, creatinine, glutamic oxaloacetic transaminase (GOT), serum glutamic pyruvic transaminase (GPT), and CEA. Fourteen ASVs assigned to the genera *Pseudomonas*, *Sporacetigenium*, *Klebsiella*, *Algoriphagus*, and *Enterococcus* were negatively correlated with GOT, whereas ASV_2334 (g_*Roseburia*), ASV_210 (g_*Oscillibacter*), and ASV_934 (g_*Ignavibacteria SJA-28*) were positively correlated with GOT. Similarly, ASV_3979 (g_*Oscillospiraceae UCG-003*), ASV_1636 (g_*Bacteroides*), ASV_1087 and ASV_1497 (g_*C. sensu stricto 1*), and ASV_2057 (g_*Roseburia*) were negatively correlated with CEA. Conversely, ASV_1617

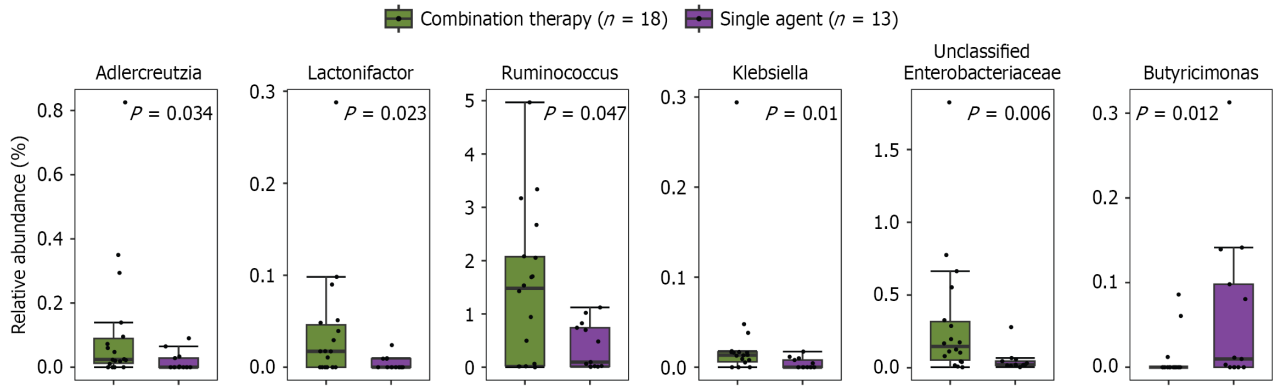

Figure 3 Bacterial genera with significantly different abundance between patient subgroups stratified by treatment regimen after chemotherapy (T2). Only genera with an uncorrected *P* value < 0.05 (Mann-Whitney *U* test) are displayed.

(g_*Streptococcus*) and ASV_80 (f_*Lachnospiraceae*) were positively correlated with CEA. Only a few associations were detected for other markers. ASV_1736 (g_*Bacteroides*) was negatively correlated with creatinine, ASV_1450 (g_*Enterococcus*) with glucose, and ASV_1444 (g_*Intestinibacter*) with GPT (Figure 4 and Supplementary Table 8).

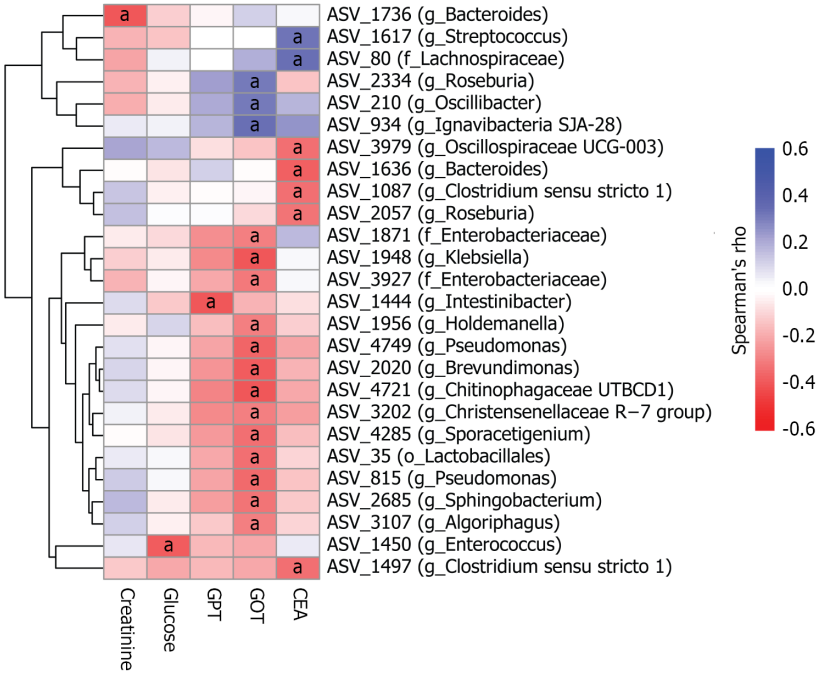

Figure 4 Heatmap showing the Spearman's rank correlations between amplicon sequence variant abundance and serum biochemical markers. Spearman's *ρ* values were computed for each amplicon sequence variant (ASV) in relation to each biochemical marker. Multiple testing was adjusted using the Benjamini-Hochberg method, and only ASVs or markers with adjusted *P* values < 0.05 are displayed. Hierarchical clustering of ASVs was performed using the Euclidean distance metric. [a]Adjusted *P* < 0.05. ASVs were labeled with genus (g_), or family (f_), or order (o_) when the genus-level classification was unavailable. ASV: Amplicon sequence variant; GOT: Glutamic oxaloacetic transaminase; GPT: Glutamic pyruvic transaminase; CEA: Carcinoembryonic antigen.

DISCUSSION

This is one of the few studies that monitored microbiota diversity and composition changes in patients with CRC during the treatment period using 16S rRNA metagenomic sequencing. Among various clinical factors, surgery and chemotherapy emerged as the primary drivers of changes in gut microbiota diversity and composition.

First, analysis of gut microbiota diversity at different timepoints revealed reduced richness and evenness after surgery (T1) and after chemotherapy (T2) compared with diagnosis (T0). These findings are consistent with the study by Png *et al* [20], showing a significant reduction in alpha diversity (Chao1 and ACE indices) in 12 Singaporean patients with CRC at month 6 after surgery. The authors showed that surgery induced substantial changes in the gut microbial community structure and diversity. Prophylactic antibiotics administered before surgery were associated with a reduction in gut microbial diversity[21]. Similarly, the gut microbial community was altered in 28 patients with non-Hodgkin's lymphoma

who received chemotherapy for 5 consecutive days[22].

When alpha diversity was examined in relation to clinical characteristics at each timepoint, no significant association was detected with most factors, except for tumor location and BMI at T1, and blood pressure and CEA concentration at T2. In a multi-cohort analysis of 546 patients, tumor location was associated with distinct alpha diversity profiles and the enrichment of specific microbial taxa linked to CRC prognosis[23]. Moreover, in patients with CRC and cachexia onset (*i.e.*, weight loss and/or a BMI < 20 kg/m^2) after surgery, alpha diversity was higher in pre-surgery stool samples, likely due to higher fiber intake[24]. Our study revealed a similar trend in underweight patients after surgery (T1). However, the participants' dietary intake was not recorded, which warrants further investigation.

Beta diversity analysis revealed qualitative changes (presence/absence of taxa) in the gut microbiota across timepoints, but not quantitative differences (relative abundance of taxa). This suggests that the post-treatment timepoints are marked by the loss of rare or low-abundance taxa rather than a restructuring of dominant communities. These results are consistent with the presence/absence analysis that showed a gradual depletion of the gut microbial community from T0 to T2. This finding reinforces previous studies showing a reduction in gut microbiota diversity following chemotherapy[22,25]. These qualitative changes indicate a progressive simplification of the gut microbial community rather than the absolute loss of specific bacterial lineages.

During cancer treatment, the gut microbial environment can be substantially altered by the combined effects of multiple interventions, such as perioperative antibiotics, bowel preparation, dietary modifications, surgery, chemotherapy, radiotherapy, and targeted therapies, rather than by any single component. These treatments may disrupt microbial community structure, leading to a reduction in beneficial taxa and an expansion of potentially pathogenic species, thus increasing the risk of inflammation, diarrhea, constipation and other gastrointestinal complications. Such dysbiosis may also diminish the treatment efficacy or exacerbate its toxicity[26,27].

In our population, the abundance of various pathogenic bacterial taxa was reduced following surgery and chemotherapy. Particularly, several genera (*e.g.*, *Parvimonas*, *Peptostreptococcus*, *Porphyromonas*, *Desulfovibrio*, *Prevotella*, and *Turicibacter*) were detected at diagnosis but declined following surgery and chemotherapy. It was previously reported that these genera are enriched in patients with CRC compared with healthy controls[13]. *Parvimonas*, particularly *Parvimonas micra*, has been linked to poorer survival in patients with CRC because it promotes colonocyte proliferation and modulates the Th17 immune response[28]. Various *Peptostreptococcus* species, such as *Peptostreptococcus anaerobius* and *Peptostreptococcus stomatis*, have been associated with chronic inflammation induction and consequently colon tumorigenesis[29,30]. The two *Porphyromonas* pathogens (*Porphyromonas gingivalis* and *Porphyromonas asaccharolytica*) enriched in patients with CRC at diagnosis have been associated with colorectal tumorigenesis through butyrate production[31]. Similarly, *Desulfovibrio desulfuricans* can convert sulfates into hydrogen sulfide that in turn, induces DNA damage and accelerates CRC progression[32]. A previous study demonstrated that *Prevotella*, together with 3-oxocholic acid, can facilitate CRC progression and counteract the FOLFOX effect[33]. Conversely, *Turicibacter* is generally considered beneficial for the host's metabolic and lipid health by modulating bile acid and lipid metabolism[34]. Other CRC-associated genera, including *Fusobacterium*, *Pseudomonas*, *Klebsiella*, *Gemella*, and *Solobacterium*, were also depleted after treatment. Many studies reported that *Fusobacterium* (specifically *F. nucleatum*) is associated with CRC pathogenesis and chemotherapy adverse effects[8,35]. The role of *Pseudomonas* in CRC remains controversial. Indeed, *Pseudomonas aeruginosa*, a common *Pseudomonas* species, contributes to the pathogenesis of cancer-related epithelial phenotypes[36], but also enhances the therapeutic efficacy of anti-programmed cell death protein 1 immunotherapy[37]. Several species from the genera *Klebsiella*, *Gemella*, and *Solobacterium*, such as *Klebsiella aerogenes*, *Gemella morbillorum* and *Solobacterium moorei*, are increased in patients with CRC and promote tumor progression[38-40].

Following treatment (surgery and chemotherapy), several beneficial gut bacterial taxa were also decreased, suggesting that standard therapies broadly disrupt the gut microbial community rather than selectively targeting tumor-associated microbes. Notably, the abundance of several SCFA-producing bacteria, including the genera NK4A214, UCG-005, and UCG-002 (family Oscillospiraceae) and also the *E. ruminantium* group and the *R. gauvreauii* group, was reduced after chemotherapy (T2). The depletion of these genera may lead to reduced SCFA production. SCFAs help to maintain the epithelial barrier and reinforce mucosal and systemic immunity, which are generally considered beneficial for gut health, particularly by reducing intestinal inflammation and improving responses to cancer therapies[41]. Therefore, the loss of these taxa may weaken the gut barrier, potentially increasing chemotherapy toxicity or reducing efficacy. However, this hypothesis requires further mechanistic investigation of the underlying metabolic and functional pathways, as these interpretations are inferred from taxonomic data and prior literature, and direct functional or metabolomic measurements were not performed in this study.

On the other hand, several genera increased after treatment. The *Lachnospiraceae NK4A136* group increased after surgery, but declined after chemotherapy. This genus is a butyrate producer and enhances the gut barrier function[42]. *Anaerotruncus*, *Megasphaera*, and *Parasutterella* were enriched after chemotherapy (T2). A higher abundance of *Anaerotruncus* has been associated with shorter overall survival and may be a marker of CRC recurrence[43]. A systematic analysis of fecal metagenomic datasets found that *Megasphaera* is consistently enriched in individuals with inflammatory bowel disease, adenoma, and CRC compared with healthy controls. This genus contributes to colon tumorigenesis by inducing dendritic cell-mediated inflammation[44]. Conversely, previous studies found that *Parasutterella* abundance is reduced in patients with CRC compared with healthy controls[13,45]. This genus promotes health and maintains the bile acid homeostasis[46], which in turn helps to reduce CRC risk[47]. The post-treatment increases in genera such as *Megasphaera* and *Parasutterella* may reflect compensatory responses to dysbiosis or chemotherapy-specific effects, requiring in-depth analysis into their roles in recovery or recurrence.

Our preliminary findings revealed several genera differ between the single-agent therapy and the combination therapy before multiple testing corrections. In particular, a higher abundance of *Klebsiella* was observed in the combination therapy compared with the single-agent therapy. Members of this genus, particularly *Klebsiella pneumoniae* (*K. pneumoniae*),

are well-recognized opportunistic pathogens frequently associated with severe infections in hospital settings. Clinical isolates of *K. pneumoniae* from malignant cancer patients possess multiple virulence factors and diverse antimicrobial resistance genes, leading to poor treatment outcomes, especially in immunocompromised cancer patients[48]. *Ruminococcus* and *Butyricimonas* are both known butyrate-producing genera but exhibited opposite trends between the two treatment regimens. *Ruminococcus* was increased in association with the combination therapy, whereas *Butyricimonas* showed higher abundance in the single-agent therapy. Butyrate has been reported to reduce gut inflammation and mitigate 5-FU toxicity[10]. The inconsistent patterns observed among butyrate-producing bacteria across treatment regimens require additional investigation, particularly through the quantification of SCFA production in relation to treatment type, to better understand therapy-associated toxicity and the role of butyrate-producing microbes. In addition to associations with treatment regimens, differences in gut microbial composition were also detected between patient subgroups stratified by treatment response. *Eubacterium* and *Oscillospiraceae UCG-005* were enriched in responders, while *Bacteroidetes vadinHA17*, *Lactococcus*, and *Subdoligranulum* were increased in non-responders. Similar trends in these taxa have been reported in melanoma patients receiving anti-PD-1 therapy when comparing responders and non-responders. Of note, Ruminococcaceae (also known as Oscillospiraceae) was positively associated with effector $CD4^+$ and $CD8^+$ T-cell levels and sustained cytokine responses during therapy, potentially enhancing anti-tumor immunity[49].

The weak correlations between bacterial ASV abundances and serum markers (*e.g.*, a positive association of *Roseburia* with GOT and a negative association with CEA) suggest potential host-microbiome interactions. For instance, *Roseburia*, a butyrate producer, may influence liver enzymes through its anti-inflammatory effects or by facilitating the transport of metabolites[50]. Meanwhile, increases in *Oscillospiraceae UCG-003*, *Bacteroides*, *C. sensu stricto 1*, and *Roseburia* linked to lower CEA levels, could indicate modulation of tumor burden. This preliminary finding is further supported by a recent study[51], which reported correlations between specific bacterial taxa and CEA levels, along with the application of machine learning models to predict blood CEA levels based on gut microbiota profiles.

This study has several limitations that should be acknowledged. First, external confounding factors, such as diet, concomitant medications, and the use of antibiotics, probiotics, or gastric protectants during treatment, were not fully controlled, although they could influence the gut microbiota composition. Particularly, perioperative antibiotic use represents a major confounding factor. However, because antibiotic prophylaxis is an integral component of standard surgical care, the observed microbiota disruption reflects real-world treatment effects rather than the isolated surgery impact. Second, the sample size was relatively small (n = 31), particularly for a longitudinal cohort. This limited the ability to conduct stratified analyses or balanced subgroup analyses by tumor stage, tumor location, regimen-specific treatment or treatment response, thereby restricting generalizability, and reducing statistical power, as well as the robustness and interpretability of our findings. However, the control of the type I error rate does not depend on the sample size. When significant associations are observed (*i.e.*, raw P value or adjusted $P < 0.05$), these findings are as statistically valid as those obtained from larger sample sizes. Therefore, the exploratory analysis suggesting potential associations between gut microbiota composition and clinical factors may serve as a preliminary foundation and require further validation in larger cohorts. Third, the study was limited to 16S rRNA sequencing, which does not provide species-level resolution or functional insights into microbial activity. Fourth, although temporal changes in microbiota were described, causal relationships with treatment efficacy or toxicity were not directly assessed. Furthermore, although sampling after surgery and after chemotherapy was conducted at least three weeks post-treatment to allow for gut microbiota recovery, these time points may still represent an acute change phase rather than a stable recovery phase. Finally, the study is limited by its within-patient longitudinal design and the absence of a healthy control group or a post-surgery non-chemotherapy control group. As a result, it is impossible to clearly distinguish which observed microbiota changes are treatment-specific *vs* attributable to the natural disease progression or surgery itself, nor to determine if the baseline state (T0) was already abnormal. Future studies that integrate shotgun metagenomics, metabolic profiling, and clinical outcomes in larger, well-controlled cohorts including a healthy control group are required to validate and extend these findings.

CONCLUSION

Surgery and chemotherapy were the principal determinants of gut microbiota changes in patients with CRC. Throughout treatment, patients exhibited significant changes in gut microbial diversity and composition. After surgery and after chemotherapy fecal samples showed reduced alpha diversity and loss of multiple taxa, including CRC-associated pathogens and SCFA-producing bacteria, particularly after chemotherapy. Collectively, these findings underscore the substantial impact of standard CRC therapies on gut microbiota composition, supporting the potential of microbiota-targeted or probiotic-based interventions as adjunctive strategies in CRC management.

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