

Differences in the on- and off-tumour microbiota between right- and left-sided colorectal cancer

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Research Article

Keywords: Microbiota, Proximal, Distal, Colorectal Cancer

Posted Date: February 11th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-226410/v1>

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Abstract

Objective This study aims to determine the differences in the microbial profiles of the on- and off-tumour microbiota between patients with right- and left-sided colorectal cancer.

Design Microbial profiling of on- and off-tumour biopsies from patients with right-sided (n=17) and left-sided (n=7) colorectal adenocarcinoma was performed using 16S ribosomal RNA sequencing.

Results Off-tumour alpha diversity is significantly greater in right-sided compared to left-sided colorectal cancer patients. However, no differences in on-tumour alpha diversity were observed between tumour locations. Off-tumour beta diversity showed distinctive bacterial community clusters between the right and left colon, while no significant differences in beta diversity were observed in the on-tumour microbiota. The off-tumour microbiota showed the right colon to be enriched with species of the *Lachnospirillum*, *Selenomonas* and *Ruminococcus* genera, whereas the left colon is enriched with Epsilonbacteraeota phylum, Campylobacteria class, and Pasteurellales and Campylobacterales orders. In contrast the on-tumour microbiota showed relatively fewer differences in bacterial taxonomy between tumour sites, with left tumours being enriched with *Methylophilaceae* and *Vadin BE97* families and *Alloprevotella*, *Intestinibacter*, *Romboutsia* and *Ruminococcus 2* genera. Comparison of paired on- and off-tumour microbiotas showed that patients with left-sided colorectal cancer had large taxonomic differences between their on- and off-tumour microbiota, while patients with right-sided colorectal cancer showed relatively fewer taxonomic differences.

Conclusion The off-tumour microbiota is more diverse in the right compared to the left colon, showing distinct community clusters and large differences in bacterial taxonomy. In contrast the on-tumour microbiota shows no difference in bacterial diversity and relatively fewer differences in bacterial taxonomy. Collectively this suggests that the right and left colon show distinctive bacterial communities, however, the presence of a colonic tumour leads to a more consistent microbiota between locations.

Trial registration number [clinicaltrials.gov \(NCT01701310\)](https://clinicaltrials.gov/ct2/show/study/NCT01701310).

Introduction

The term colorectal cancer encompasses a heterogeneous group of tumours of the lower gastrointestinal tract. These include those of the caecum, ascending and transverse colon, termed right-sided colorectal cancer, and those of the descending colon, sigmoid colon, and the rectum, termed left-sided colorectal cancer.^{1,2} Tumours of the right and left colon have distinctive developmental and physiological differences, leading to variability in disease outcomes, prognosis, and response to therapy.^{3,4} Further understanding of disease initiation and progression in right- and left-sided colorectal cancer can aid in disease prevention and treatment.

A multistep pathogenesis is involved in the aetiology of sporadic colorectal tumours, with genetics, lifestyle and environment are all known to contribute to colorectal carcinogenesis.⁵ Along with this the gut

bacterial microbiota and its metabolites have also been shown to both promote and protect against colorectal cancer.⁶ Therefore, identifying the differences in bacterial populations between tumour-associated (on-tumour) and tumour-adjacent (off-tumour) microbiota and how they differ between the right and left colon has the potential to be beneficial in predicting disease outcomes and aid in patient stratification.^{7,8}

A previous study by Flemer *et al*/ showed that the microbiota of healthy patients, those with polyps and with colorectal cancer were significantly different. The authors also determined that the on- and off-tumour microbiota shows no differences in microbial communities, whereas the on-tumour microbiota from the right and left colon were significantly different.⁹ However, as subsequently discussed by Al-Hassi *et al*, right-sided colorectal tumours are more commonly associated with iron deficiency anaemia relative to left-sided tumours, hence more often requiring iron therapy.¹⁰ The use of enteral iron supplementation was not assessed by the authors in the Flemer study and has the potential to be responsible for differences observed.⁹ Enteral iron supplementation have the potential to alter the bacterial microbiota through increasing gut luminal iron availability for bacterial cell proliferation. Hence, this suggests the use of parental iron supplementation in anaemic colorectal cancer patients in order to study the gut microbiota.^{11,12}

This pilot study aims to determine the gut bacterial profiles of the on- and off-tumour microbiota in colorectal cancer and how they differ between patients with right- and left-sided colorectal cancer. In order to address the issue regarding iron supplementation, all patients were treated with parental iron prior to surgery.

Methods

Study population and sample collection

24 anaemic patients with non-metastatic histologically proven colorectal adenocarcinoma, presenting with right-sided (n=17) and left-sided (n=7) tumours, from the intravenous iron in colorectal cancer associated anaemia (IVICA) trial were included in the study. All patients received intravenous iron (ferric carboxymaltose - Ferinject™; Vifor Pharma, Glattbrugg, Switzerland) prior to surgery, dosed by weight and haemoglobin in accordance with the summary of product characteristics.¹³ Treatment was administered at least two weeks preoperatively and anaemia was defined as having a haemoglobin level 10 g/l below the sex-specific World Health Organisation definition (women \leq 120 g/l, men \leq 130 g/l). Patients were excluded from the study if they were currently receiving chemotherapeutic treatment, had pre-existing anaemia prior to colorectal cancer diagnosis or pre-existing haematological disease. Detailed patient demographics can be found in table 1. Colorectal tumour biopsies and paired tumour-adjacent colonic mucosal tissue biopsies were obtained post-surgery.

DNA extraction and 16S ribosomal RNA amplicon sequencing

Bacterial DNA was obtained using a modified protocol of Qiagen All Prep DNA/RNA Mini Kit (Qiagen, Hilden, Germany). Colorectal tumour and paired tumour-adjacent biopsies were mechanically lysed using 5mm steel bead (Qiagen) and 0.1mm Zirconia/Silica beads (Strateck, Suffolk, UK) with a TissueLyser (Qiagen), followed by enzymatic and heat lysis. Extracted microbial DNA was used for 16S ribosomal RNA (rRNA) gene amplification and sequencing to determine the mucosal-adherent microbiota according to the Earth Microbiome project protocol.¹⁴ Using primers targeted to the V4 region (515F-806R), the 16S rRNA genes were amplified in technical triplicates. This was performed using a single-step, single-indexed polymerase chain reaction (PCR). DNA extraction and 16S rRNA gene PCR were both performed in batch with appropriate multiple-reagent based negative controls. Paired-end sequencing (2x250 base pairs) was completed in a single batch using the Illumina MiSeq platform (Illumina, San Diego, USA).

Statistical analysis

Microbial bioinformatic analysis was achieved using the Quantitative Insight Into Microbial Ecology 2 (QIIME2) pipeline.¹⁵ High-quality reads were clustered into operational taxonomic units (OTUs), reads with a 97% sequence identity were allocated to a single OTU and were assigned bacterial taxonomy using the Silva-132-99% OTU database.¹⁶ Alpha diversity was assessed using Mann-Whitney U test comparing variation in Abundance-based Coverage Estimator (ACE), Chao1, and observed OTUs between groups. Beta diversity was assessed using permutational multivariate analysis of variance (PERMANOVA) comparing Jaccard similarity coefficients, with distances between groups plotted using principal coordinate analysis (PCoA). PCoA and alpha diversity metrics were mapped using the R package "ggplot2".¹⁷ Comparison of relative abundances of taxa between locations and sample types were assessed using a linear discriminant analysis (LDA) effect size (LEfSe). LEfSe uses a non-parametric Kruskal-Wallis rank-sum test to identify taxa with significantly different normalised relative abundances and performs an LDA to determine an effect size of each taxa. Taxa with an LDA greater than 2 with a p-value ≤ 0.05 were considered significant.¹⁸

Results

On- and off-tumour bacterial diversity between right and left colon

A total of 4.4 million reads (109,122 reads/sample) were obtained following quality control, with a sampling depth of 8,000 reads/sample. Comparison of alpha diversity metrics shows that the off-tumour microbiota of patients with right-sided colorectal cancer showed significantly greater bacterial abundance (ACE), species richness (Observed OTUs) and bacterial diversity (Chao1), compared to the off-tumour microbiota of patients with left-sided colorectal cancer (Figure 1; $p < 0.05$). Consistent with this Jaccard similarity assessed beta diversity, showing that the off-tumour microbiota of right-sided and left-sided colorectal cancer patients formed significantly distinct bacterial community clusters (Figure 2; $p < 0.05$).

In contrast the on-tumour microbiota showed no differences in alpha diversity between right- and left-sided colorectal cancer patients (Figure 1; *ns*). Furthermore, beta diversity analysis showed that right- and

left-sided tumours showed no significant differences in Jaccard similarity (Figure 2; *ns*). Collectively this suggests that bacterial diversity is greater in the right colon compared to the left, however, the presence of a colonic tumour leads to a more consistent bacterial diversity between locations.

Gut phylogenetic profiles between right- and left-sided colorectal cancer patients

Firmicutes, Bacteroides, Proteobacteria and Fusobacteria constitute >95% of bacteria phyla in each group, showing largely consistent phylum relative abundance between locations (Figure 3a). The 3 dominant bacterial families across all locations are *Lachnospiraceae*, *Ruminococcaceae* and *Bacteroidaceae*. However, there is variation in the subsequent most abundant families between locations. Within the off-tumour microbiota the next most abundant family was *Fusobacteriaceae*, followed by *Enterobacteriaceae* in the right colon and *Prevotellaceae* in the left colon. While in the on-tumour microbiota the next most abundant family is *Prevotellaceae*, followed by *Streptococcaceae* in the right-sided tumour and *Rikenellaceae* in the left-sided tumours (Figure 3b).

Comparison of bacterial taxa between right and left colon

Differences in gut bacterial populations between the right- and left-sided colorectal cancer patients were assessed using LEfSe to determine bacterial taxa that are significantly enriched between locations. The off-tumour microbiota of right-sided colorectal cancer patients showed greater abundances of species of the *Lachnoclostridium*, *Selenomonas* and *Ruminococcus* genera. Whereas, the off-tumour microbiota of left-sided colorectal cancer patients is enriched with Epsilonbacteraeota phylum, Campylobacteria class, Pasteurellales and Campylobacterales orders, *Campylobacteraceae*, *Bacillales Family XI*, *Clostridiales Family XI*, *Peptostreptococcaceae* and *Pasteurellaceae* families and *Campylobacter*, *Gemella*, *Granulicatella*, *Parvimonas*, *Anaerosporebacter*, *Lachnospiraceae* (UCG010), *Peptostreptococcus*, *Selenomonas* and *Haemophilus* genera (Figure 4a, b).

The on-tumour microbiota of left-sided cancer patients showed greater abundances of *Methylophilaceae* and *Vadin BE97* families and *Alloprevotella*, *Intestinibacter*, *Romboutsia* and *Ruminococcus 2* genera, compared to the on-tumour microbiota of right-sided colorectal cancer patients (Figure 4c, d). These results support the diversity data, with the off-tumour microbiota showing large differences in bacterial populations between the right and left colon. In contrast, the on-tumour microbiota seems less affected by location, supporting a cancer defined microbiota that is more constant between the right and left colon.

Difference in paired on- and off-tumour bacterial taxa in right- and left-sided colorectal cancer patients

In order to assess the differences in bacterial taxa between the tumour-associated and tumour adjacent microbiotas in right- and left-sided colorectal cancer patients, we performed an LEfSe comparing paired on- and off-tumour bacterial taxa in each location. In the right-sided colorectal cancer patients there were 24 bacterial taxa that were differentially enriched between the on- and off-tumour microbiota. These include the *Lachnoclostridium* genus which was enriched in the on-tumour microbiota and the

Cyanobacteria phylum, Melainabacteria class, Gastranaerophilales and Corynebacteriales orders, *Dietziaceae*, *Corynebacteriaceae*, *Eggerthellaceae*, *Rikenellaceae* and *Clostridiales vadin BB60 group* families, *Dietzia*, *Paraprevotella*, *Prevotella 9*, *Alistipes*, *Lachnospira*, *Ruminococcus torques group*, *Paeniclostridium*, *Eubacterium coprostanoligenes group*, *Acidaminococcus* and *Aquabacterium* genera which were enriched in the off-tumour microbiota (Figure 5c, d). In the left-sided colorectal cancer patients there were 3 bacterial taxa differentially enriched between the on- and off-tumour microbiota. These include the *Porphyromonadaceae* family and *Lachnospira* and *Porphyromonas* genera, which were more abundant in the on-tumour compared to the off-tumour microbiota (Figure 5a, b). Collectively this suggests that patients with right-sided colorectal cancer have an on- and off-tumour microbiota that is relatively consistent, showing only small differences in bacterial taxa lower taxonomic levels. In contrast, patients with left-sided colorectal cancer have an on- and off-tumour microbiota that show distinct bacterial populations, showing differences at the phylum, class, and order taxonomic levels.

Discussion

Previous studies have attempted to unravel the complex relationship between the gut bacterial microbiota and colorectal cancer.^{8,9} However, the composition of the gut microbiota can be influenced by a multitude of environmental variables, such as diet and medication. Hence, this leaves the potential for the study of the gut microbiota in pathological conditions to be confounded by discrepancies in therapeutic interventions.^{11,12} For instance, patients with right-sided colorectal cancer tend to more commonly develop iron deficiency anaemia, compared to those with left-sided colorectal cancer. Enteral iron is often given to treat anaemia, which has the potential to increase colonic iron concentration and alter the gut microbiota.¹⁰ Therefore, this has the possibility for studies comparing the microbiota between right- and left-sided colorectal cancer patients to be confounded by an unequal prevalence of iron deficiency anaemia and iron therapy between cohorts. This pilot study aimed to determine the differences in on- and off-tumour microbiota between right- and left-sided colorectal cancer patients, while ensuring consistency in the prevalence of iron deficiency anaemia and iron therapy. In order to overcome the potential for enteral iron influencing gut bacterial populations, all patients received parenteral iron prior to surgery.

This study shows that the on- and off-tumour microbiota between right- and left-sided colorectal cancer patients show differential microbial diversity and bacterial taxa. Off-tumour alpha diversity is significantly greater in right- compared to left-sided colorectal cancer patients, showing greater bacterial diversity, abundance, and richness. Furthermore, off-tumour beta diversity shows significantly different bacterial community clusters between right- and left-sided colorectal cancer patients. Comparison of off-tumour taxonomy shows there to be 29 bacterial taxa that are differentially enriched between the right and left colon. These differences in off-tumour bacterial populations between the right and left colon are potentially explained by differences in colonic nutrient availability. Nutrient availability is greatest in the proximal colon and decreases towards the distal colon, through nutrients being reabsorbed in the colon and being utilised by residential bacteria.¹⁹ Therefore, this potentially creates differential nutrient niches

available for colonic bacteria between the right and left colon, which may explain why the right colon shows greater bacterial diversity compared to the left.²⁰

In contrast the on-tumour microbiota shows no differences in alpha and beta diversity between right- and left-sided colorectal cancer patients. Comparison of on-tumour taxonomy shows there to be 8 bacterial taxa that are differentially enriched between the right and left colon. Collectively this suggests that the on-tumour microbiota between right- and left-sided colorectal cancer patients are relatively more consistent, when compared to the differences observed in the off-tumour microbiota. Suggesting that the on-tumour microbiota between the right and left colon is less affected by colonic nutrient availability, potentially supporting a cancer defined microbiota that is less affected by tumour location.

Comparison of bacterial taxa between paired on- and off-tumour microbiota showed that patients with right-sided colorectal cancer had a relatively consistent on- and off-tumour microbiota, with only 3 bacterial taxa being differentially enriched. In contrast patients with left-sided colorectal cancer had a more varied on- and off-tumour microbiota, showing 24 differentially enriched bacterial taxa. This potentially suggests that the on-tumour microbiota found in the right and left colon is more similar to the off-tumour microbiota found in the right colon compared to the left. Suggesting that in the left colon there is a shift in the on-tumour microbiota away from the off-tumour microbiota, becoming more similar to the right colonic microbiota.

This pilot study provides a novel insight into the on- and off-tumour microbial profiles between right- and left-sided colorectal cancer patients. Suggesting a varied microbiota between the right and left colon, however, the presence of a colonic tumour leads to a more consistent cancer defined microbiota. This is potentially through the left tumour microbiota being more similar to the microbiota found in the right colon, suggesting that the right colonic microbiota may be more favourable to support colonic tumours. Despite the relatively small sample size of this study, we were able to infer significant differences between right- and left-sided colorectal cancer patients, which can form the foundation for large-scale explorative studies. Further understanding of the gut bacterial microbiota in colorectal cancer and how it may vary depending upon colonic location, can assist in understanding the role of the microbiota in gut pathology and aid in patient stratification for clinical trials and potential probiotic therapies.

Declarations

Ethical Approval

Ethical approval for the IVICA trial was granted by the National Research and Ethics Service (East Midlands - Nottingham 2 Research Ethics Committee, 11/EM/0237). The study was registered with Clinical Trials.Gov (NCT01701310) and the Medicines and Healthcare Products Regulatory Agency (2011-002185-21). The study was undertaken in line with the Declaration of Helsinki.

Competing Interest

MJB's research department has received grant support from Tillots Pharma and Vifor Pharma (Switzerland). MJB has received honoraria and travel support for consulting or lecturing from Vifor Pharma, Tillots Pharma, and Abbvie. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Figures

	Right-sided Colorectal Cancer	Left-sided Colorectal Cancer
<i>N</i> =	17	7
Age	75.3 [7.5]	74 [7.7]
Male	9 (53%)	7 (100%)
Female	8 (47%)	0 (0%)
Height, m	1.67 [0.08]	1.77 [0.08]
Weight, kg	75.6 [12.8]	88.5 [24.0]
Inclusion Hb, g/l	97.1 [14.1]	102.9 [10.0]
Recruitment ferritin, µg/l *	25 [15-55]	10 [8-49]
Recruitment transferrin saturation, % *	2.6 [2.3-3.6]	2.8 [2.6-3.3]
Duration of iron treatment, days *	21 [15-35]	28 [15-43]
Tumour Stage		
T≤2	1 (6%)	0 (0%)
T3	3 (18%)	5 (71%)
T4	13 (76%)	2 (29%)
Preoperative Risk Assessment		
ASA fitness status classification		
I-II	7 (41%)	2 (29%)
III-IV	10 (59%)	5 (71%)
CR-POSSUM mortality score, % *	3.5 [2.5-9.3]	3.5 [2.6-6.6]

Figure 1

Patient cohort demographics. Categorical variables are presented with percentages. Continuous variables are presented as mean value [standard deviation] or *median value [interquartile range]. Hb, haemoglobin. CR-POSSUM, ColoRectal Physiological and Operative Severity Score for the enumeration of Mortality and morbidity. ASA, American Society of Anaesthesiologist.

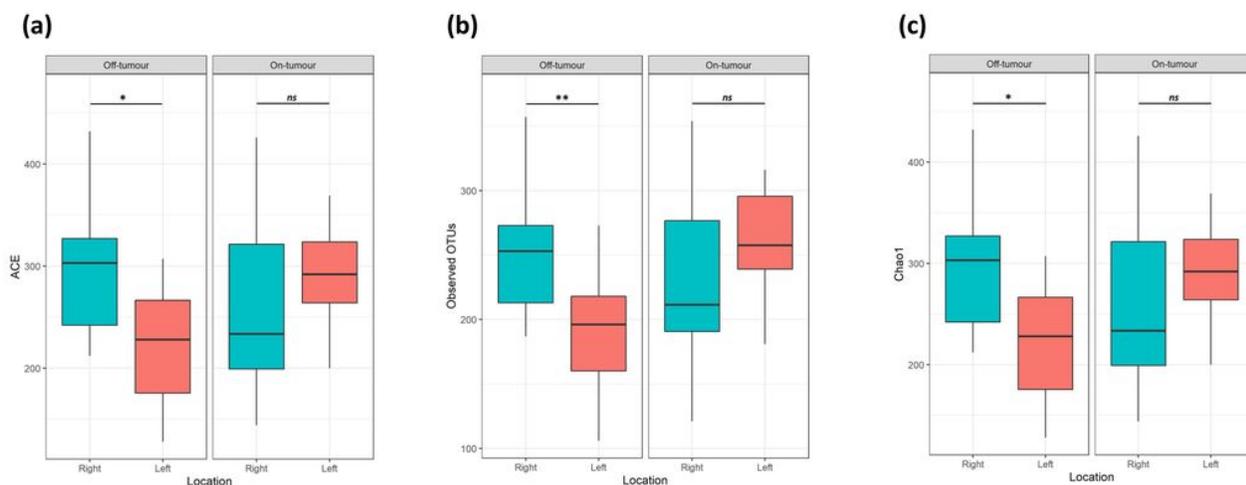


Figure 2

On- and off-tumour alpha diversity between right and left colon. Alpha diversity metrics (a) Abundance-based coverage estimate (ACE), (b) Observed operational taxonomic units (OTUs) and (c) Chao1 were significantly greater in the right compared to the left off-tumour microbiota. On-tumour alpha diversity metrics showed no significant differences between tumour location. (* $p \leq 0.05$, ** $p \leq 0.01$, ns $p > 0.05$)

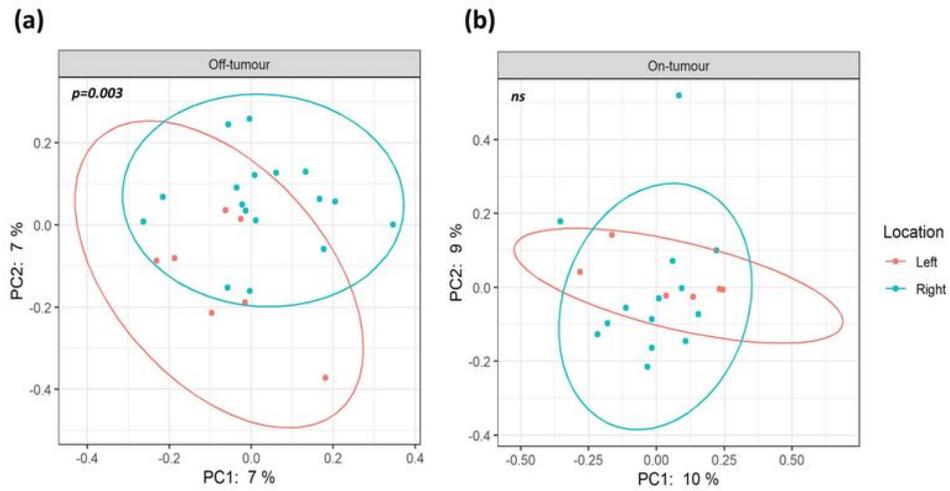


Figure 3

On- and off-tumour beta diversity between right and left colon. Principle coordinate analysis (PCoA) plots based on Jaccard distances show significantly distinct bacterial community clusters ($p=0.003$) between the off-tumour microbiota from the right and left colon (a). On-tumour microbiota from the right and left colon shows no significant differences (ns) (b).

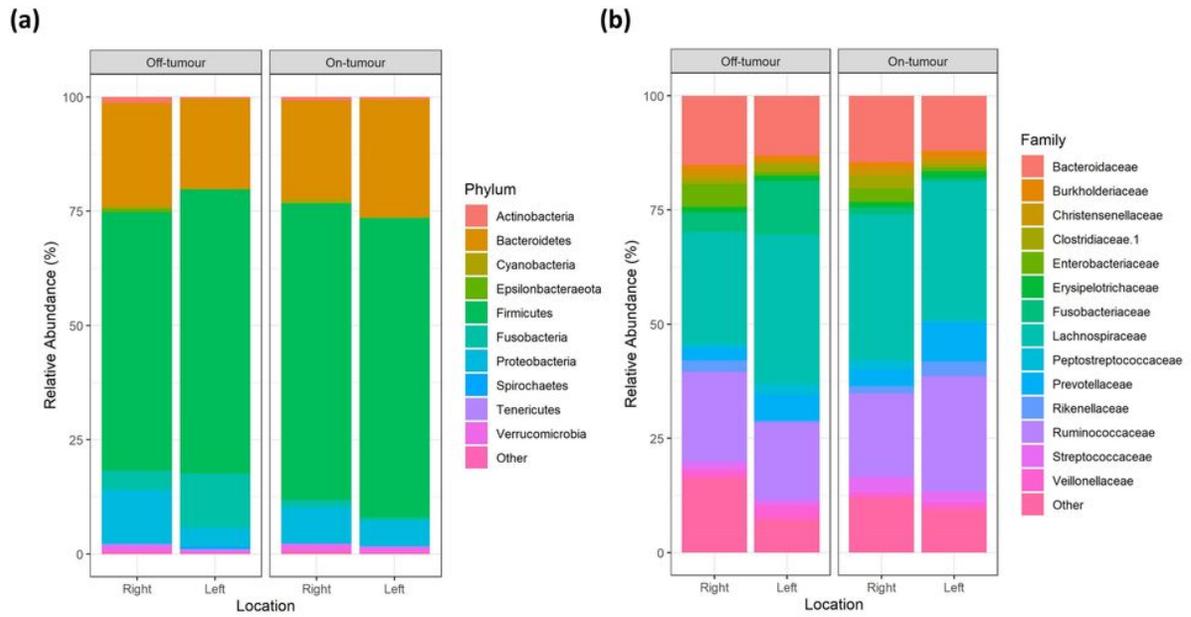


Figure 4

On- and off-tumour phylogenetic profiles of gut bacterial populations in the right and left colon. Relative abundance of on- and off-tumour mucosal adherent gut bacterial microbiota at phylum (a) and family (b) taxonomic levels in right and left colon.

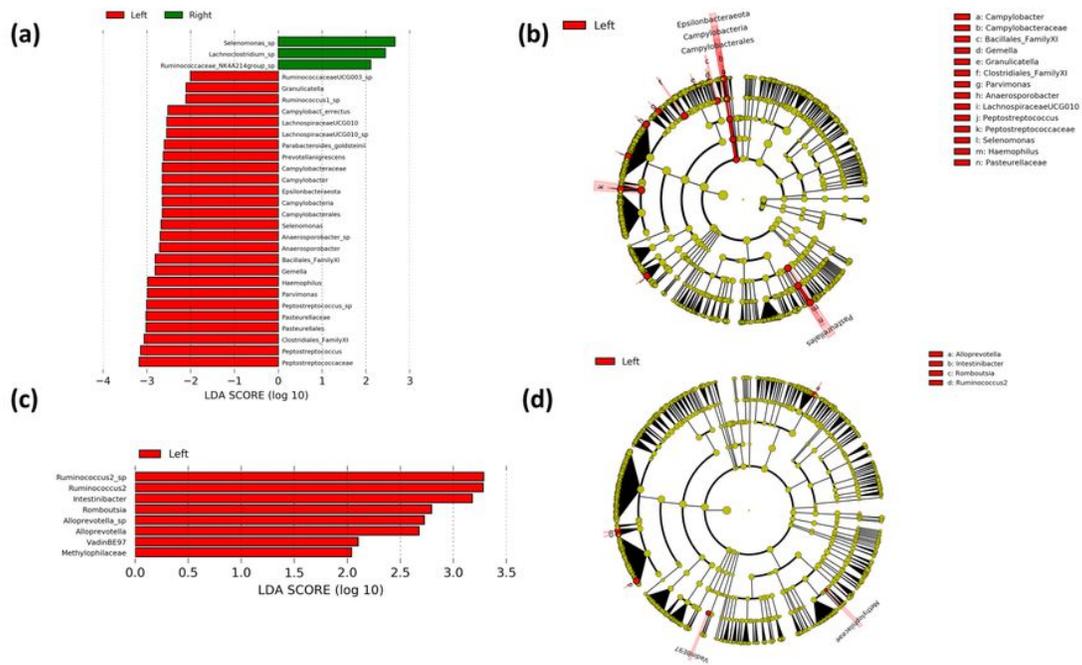


Figure 5

Linear discriminant analysis (LDA) effect size (LEfSe) comparing right and left bacterial taxa in the on- and off-tumour microbiota. Histograms of LDA scores for differentially abundant bacterial taxa between right and left colon in off-tumour (a) and on-tumour (c). LEfSe cladogram representing differentially abundant bacterial groups in off-tumour (b) and on-tumour (d) microbiota between right and left colon. Differentially abundant taxa at the genus taxonomic levels or higher were included. Taxa and nodes highlighted in green were more significant in the right colon and red in the left colon.

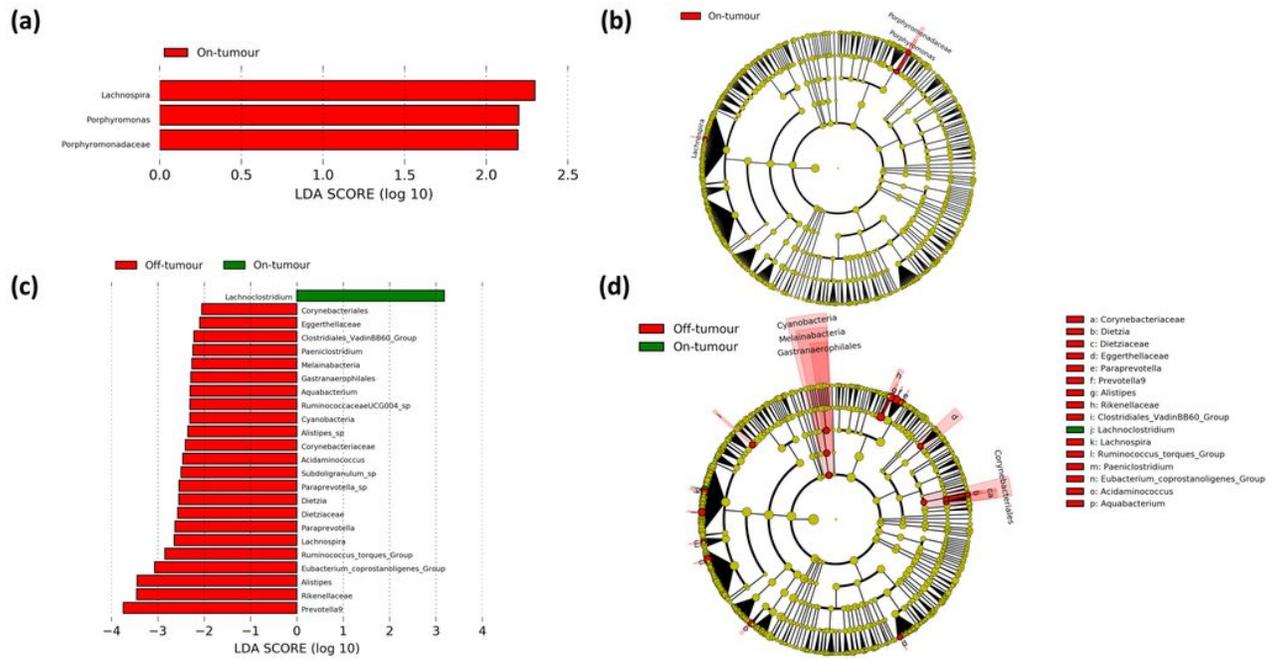


Figure 6

Comparison of paired on- and off-tumour microbiota between right and left colon. LDA scores for differentially abundant bacterial taxa between paired on- and off-tumour microbiota in right (a) and left (c) colon. LefSe cladogram demonstrating differentially abundant bacterial taxa between paired on- and off-tumour microbiota in right (b) and left (d) colon. Differentially abundant taxa at the genus taxonomic levels or higher were included. Taxa and nodes highlighted in red were more significant in the off-tumour microbiota and green in the on-tumour microbiota.