Original article

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Alterations in blood microbiota after colonic cancer surgery

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Background: **Mechanisms contributing to the perioperative stress response remain poorly understood. This study investigated changes in the amount of bacterial DNA in blood and the diversity of blood microbiota in the perioperative period in patients undergoing minimally invasive surgery for colonic cancer in an enhanced recovery after surgery setting.**

Methods: **DNA encoding the bacterial 16S ribosomal RNA gene (16S rDNA) in whole blood obtained the day before surgery, and on postoperative day (POD) 1 and POD 10–14 was amplified and quantified by PCR before sequencing for taxonomic assignment. Richness, evenness and similarity measures were calculated to compare microbiota between days. Differences in relative abundance were analysed using the linear discriminant analysis effect size (LEfSe) algorithm.**

Results: **Thirty patients were included between January and July 2016. The concentration of bacterial 16S rDNA in blood increased between the day before surgery and POD 1 ($P = 0.025$). Bacterial richness was lower on POD 10–14 than on the day before surgery and POD 1 (both $P < 0{\cdot}001$). LEfSe analysis comparing the day before surgery and POD 10–14 identified changes in the abundance of several bacteria, including *Fusobacterium nucleatum*, which was relatively enriched on POD 10–14.**

Conclusion: **These findings suggest that the blood of patients with colonic cancer harbours bacterial 16S rDNA, which increases in concentration after surgery.**

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Introduction

Globally, colorectal cancer is a major contributor to morbidity and mortality, with more than 1·2 million new cases and more than 600 000 colorectal cancer-related deaths worldwide every year[1]. Surgical resection of the tumour and associated mesenteric lymph nodes is the mainstay of treatment with curative intent[2]. Even though short-term morbidity and mortality rates have decreased in recent years as a result of advancements in surgical and oncological treatment, recurrence is still a major concern, developing in one-third of patients with UICC stage III colorectal cancer[3,4].

There is growing evidence to indicate that the stress response to anaesthesia and surgery influences the risk of recurrence after cancer surgery[5,6]. The perioperative stress response is characterized locally by acute tissue inflammation and increased levels of growth and angiogenic factors, and systemically by increased levels of catecholamines, inflammatory mediators and increased platelet activation. The initial proinflammatory response is followed by a longer period of immunosuppression, all together creating an optimal tumour-promoting environment[7–10].

Several perioperative stress modulators have been identified, such as the extent of tissue injury, pain, anxiety, hypothermia, metabolic disturbances and blood product transfusion[11]. The stress response can induce translocation of bacteria from the gut to the bloodstream owing to impaired mucosal barrier function and the surgical

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intervention itself (manipulation, dissection, division, ligation of vessels and anastomosis)[12,13]. Previous studies demonstrated translocation of bacteria from the gut to mesenteric lymph nodes in 15 per cent of patients undergoing laparotomy. Bacterial lymph node positivity was associated with septic complications, and reduced disease-specific and disease-free survival in patients with colorectal cancer[14,15]. Furthermore, clinically suspected infection requiring blood cultures within 30 days of surgery for colorectal cancer has been associated with poorer oncological outcomes[16].

Although human blood has traditionally been considered a sterile compartment without proliferating microbes, recent studies[17–19] have demonstrated that blood from healthy donors harbours a viable and rich microbiota, defined as the assemblage of microorganisms present in a defined environment. Through advances in DNA sequencing technologies, which allow comprehensive, rapid and culture-independent methods of revealing bacterial DNA, blood microbiota has been associated with several diseases with inflammatory components, such as type 2 diabetes, liver fibrosis and cardiovascular disease[20–22]. However, because of the high sensitivity of DNA sequencing technology and small amount of bacterial DNA, studying the blood microbiota is challenged by the risk of contamination by bacterial DNA from the skin, study reagents, and the environment during extraction and sequencing. Therefore, robust methods must be applied to limit the impact of contamination on study results[23,24].

The present study aimed to investigate quantitative and qualitative changes in the blood microbiota during the perioperative period of minimally invasive colonic resection for colonic cancer, with standardized care in the form of enhanced recovery after surgery (ERAS).

Methods

This was an exploratory analysis based on blood samples collected for a prospective single-centre observational study of perioperative immunological function and oxidative stress in patients with colonic cancer[25,26]. Criteria for inclusion were: diagnosis of stage I–III colonic cancer according to the UICC, and minimally invasive surgery defined as laparoscopic or robot-assisted laparoscopic surgery. Patients who had a history of previous cancer, those with known immune defects, patients undergoing neoadjuvant radiotherapy or chemotherapy, those with a postoperative histological diagnosis of benign tumour and patients with infectious postoperative complications were excluded from the study. All available patients from the original observational study[25,26] were considered eligible for this exploratory analysis. Because of the limitation of the study population imposed by the observational study and lack of previous literature for estimation of an effect size, no formal power calculation was undertaken. Patients were not involved in the design of the study, nor in the writing or editing of this paper. The Central Committee for Health Research and Ethics (file number 2008-58-0020) and the Danish Data Protection Agency (protocol SJ567) approved all analyses performed in the present study, which was conducted in accordance with the ethical guidelines of the 1975 Declaration of Helsinki. All reporting was done in accordance with the STROBE statement[27].

Perioperative course

Patients were enrolled at the Department of Surgery, Zealand University Hospital, in Roskilde, Denmark. The institution is a tertiary colorectal cancer surgical unit performing more than 300 minimally invasive colorectal procedures annually and with a fully implemented ERAS protocol[28]. The perioperative care programme has been described in detail previously[29]. All patients received information regarding the procedure, admission and recovery from doctors, nurses, physiotherapists and dieticians 4–10 days before admission. Patients were admitted on the evening before surgery. All patients undergoing left hemicolectomy and sigmoidectomy received bowel preparation in the form of an enema during the night and morning before surgery. The remaining patients had no mechanical bowel preparation before surgery and no study patient received oral antibiotics as bowel preparation. Patients received a 250-ml carbohydrate beverage at 06.00 hours on the day of surgery.

General anaesthesia comprised either propofol-based total intravenous anaesthesia or inhalational anaesthesia at the discretion of the anaesthetist. All patients received a single intravenous prophylactic dose of 240 mg gentamicin and 1 g metronidazole on induction of anaesthesia. All procedures were minimally invasive laparoscopic or robot-assisted laparoscopic operations. Principles of complete mesocolic excision with central vascular ligation were applied in all patients[30]. Ondansetron was routinely administered intravenously as prophylaxis against postoperative nausea and vomiting, whereas dexamethasone was administered only in those with a high risk of postoperative nausea and vomiting as assessed by the anaesthetist. Transurethral urinary catheters and nasogastric tubes were placed during surgery. The nasogastric tube was removed after extubation and the urinary catheter was removed in the postoperative anaesthesia care unit. Mobilization and enteral nutrition were initiated on return to the ward, and patients were

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instructed to use chewing gum for a minimum of 5 min three times a day. After surgery, 4 g paracetamol and 2 g magnesium oxide were administered daily. Opioids were administered only on demand, and nausea was treated with oral ondansetron as required.

Sample collection, DNA extraction and quantification

DNA from blood samples was isolated and amplified in a strictly controlled environment at Vaiomer (Labège, France) using a stringent contamination-aware approach, as described previously[19,23,24,31]. Blood samples were collected on the day before surgery, on postoperative day (POD) 1 and POD 10–14. Before venous puncture, the skin was wiped with ethanol. In total, 67 ml blood was collected in different tubes. The order in which the tubes were drawn was not standardized. For the present study, 9 ml blood was obtained in an EDTA tube. Within 30 min after sampling, 1·5 ml whole blood was transferred into Eppendorf tubes for quantification and profiling of DNA encoding the bacterial 16S ribosomal gene (16S rDNA). These samples were kept in a freezer at −80° C pending shipment to Vaiomer for analysis. The large volume of blood withdrawn prevented any contamination by the skin microbiome and clinical environment from having any significant impact on the results[19,23,31].

Total DNA was extracted from 100 µl whole blood using a specific Vaiomer protocol designed to minimize any risk of contamination between samples or from the researchers or environment[19]. The quality and quantity of extracted nucleic acids were controlled by gel electrophoresis and absorbance spectroscopy using a NanoDrop™ 2000 UV spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). PCR amplification was performed using 16S universal primers targeting the V3–V4 region of 16S rDNA (Vaiomer universal 16S primers). The quantitative PCR (qPCR) step was done on a VIIA 7® PCR system (Life Technologies, Carlsbad, California, USA) using Sybr Green technology. The absolute number of copies of 16S rDNA was determined by comparison with a quantitative standard curve of 16S rDNA plasmids generated by serial dilution of plasmid standards (Vaiomer Universal standard plasmids). The specificity of all qPCR products was assessed by systematic analysis of the post-PCR dissociation curve obtained between 60 and 95°C.

16S ribosomal RNA gene sequencing

The microbial population present in the samples was determined using next-generation high-throughput sequencing

Table 1 Patient characteristics

	No. of patients* (n = 30)
Age (years)†	67·6(8·8)
Sex ratio (M : F)	19 : 11
ASA grade	
I	3
II	24
III	3
BMI (kg/m^2)	
< 18·5	1
18·5–24·9	12
25·0–30·0	8
> 30·0	9
Smoking status	
Current smoker	5
Former smoker	13
Never smoker	12
Alcohol use (units/week)‡	
≤ 14 (women)/≤ 21 (men)	25
> 14 (women)/> 21 (men)	5
Charlson Co-morbidity Index score	
0	18
1	6
2	3
Missing	3
ECOG performance status score§	
0	25
1	3
2	2
UICC tumour stage	
I	10
II	12
III	8
Anaesthesia	
Intravenous	20
Inhalational	10
Operative technique	
Laparoscopy	25
Robot-assisted laparoscopy	5
Operative procedure	
Right hemicolectomy	9
Transverse colectomy	1
Left hemicolectomy	1
Sigmoidectomy	18
Total colectomy	1
Mean blood loss (ml)	36
Duration of procedure (min)†	160(53)

*Unless indicated otherwise; †values are mean(s.d.). ‡One unit is defined as 12 g ethanol. §Eastern Cooperative Oncology Group (ECOG) score ranges from 0 (fully active) to 5 (dead); a score of 0 indicates ability to carry on all predisease activities without restriction.

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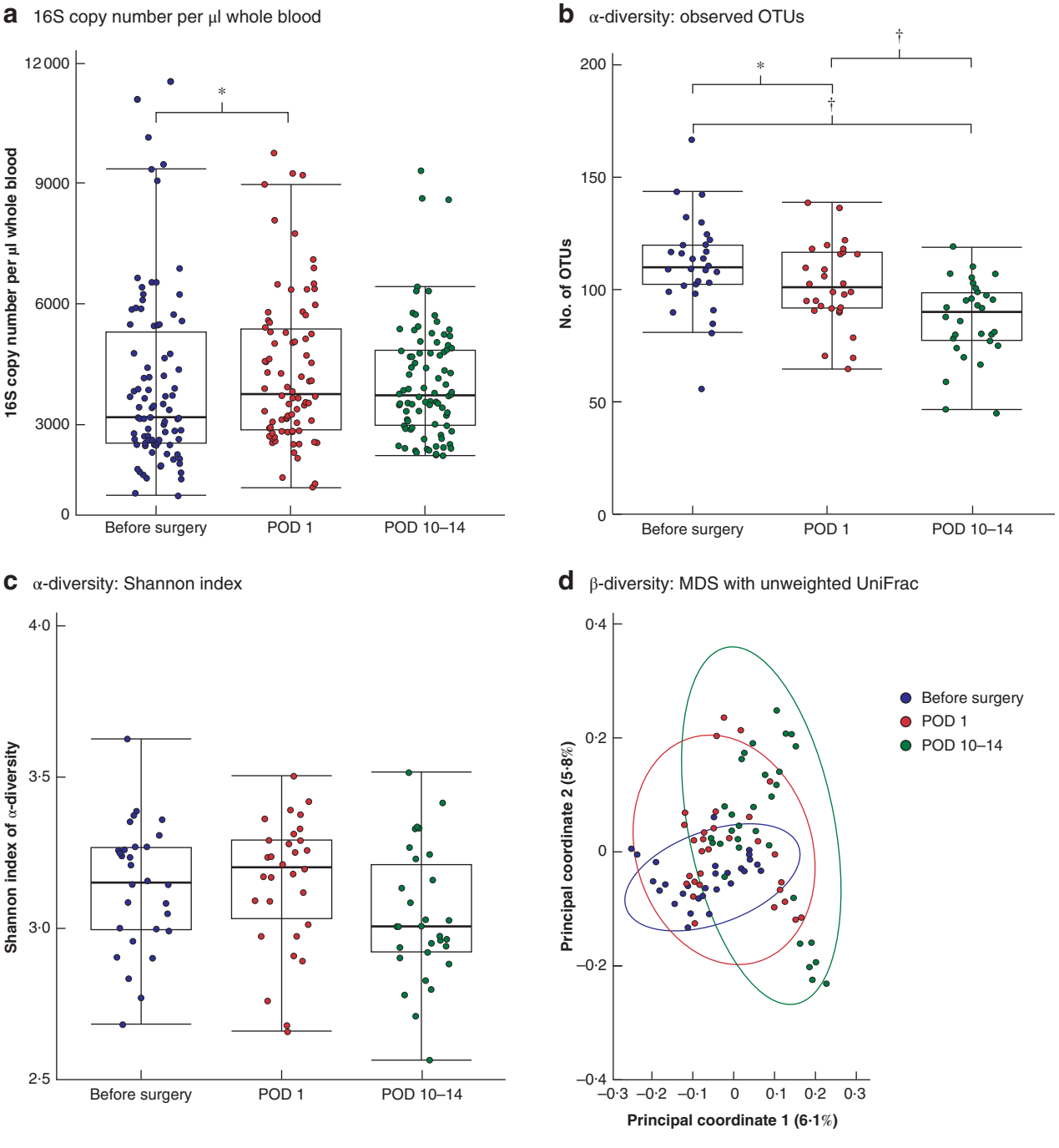
Fig. 1 Bacterial rDNA copy number, and α- and β-diversity before and after surgery



a Concentration of bacterial 16S rDNA copies per µl whole blood on the day before surgery, postoperative day (POD) 1 and POD 10–14; **b** α-diversity in terms of bacterial richness of operational taxonomic units (OTUs); **c** α-diversity regarding OTU evenness on the Shannon index; and **d** multidimensional scaling (MDS) of OTU β-diversity based on unweighted UniFrac methodology. In **a**–**c** individual values are shown, along with median (bold line), i.q.r. (box) and range (error bars). *$P < 0{\cdot}050$, †$P < 0{\cdot}001$ (**a** linear-mixed effect model; **b**,**c** paired Student's *t* test).

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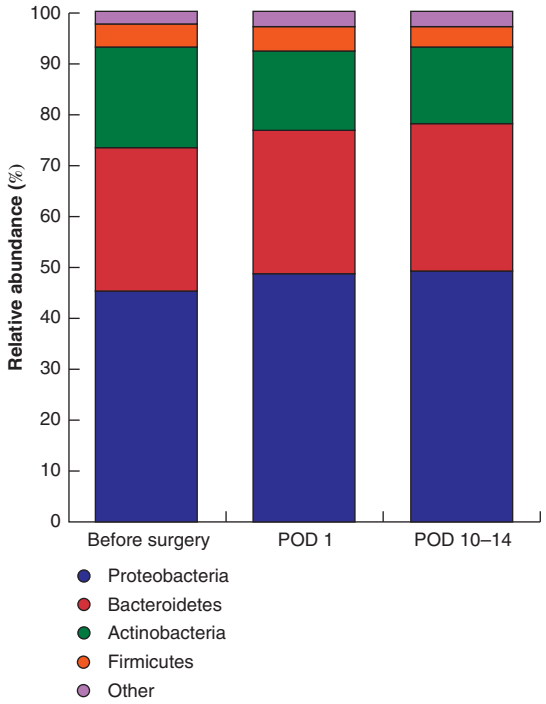
of V3–V4 variable regions of the 16S rRNA bacterial gene as described by Lluch and colleagues[24]. For each sample, a sequencing library was generated by addition of sequencing adapters. The joint pair length was set to encompass a 467-base pair amplicon (using *Escherichia coli* 16S as a reference) with a 2 × 300 paired-end MiSeq kit V3 (Illumina, San Diego, California, USA). The sequencing fragments were detected using MiSeq Illumina® technology.

Targeted metagenomic sequences from microbiota were analysed using the bioinformatic pipeline from the FROGS (Find, Rapidly, OTUs with Galaxy Solution) guidelines[32]. Briefly, the cleaning was done by removing amplicons without the two PCR primers (10 per cent of mismatches were authorized), amplicons with at least one ambiguous nucleotides (N), amplicons identified as chimera (with vsearch v1.9.5), and amplicons with a strong similarity (coverage and identity at least 80 per cent) with the phiX library (used as a control for Illumina sequencing runs). Clustering was produced in two passes of the swarm algorithm v2.1.6. The first pass was a clustering with an aggregation distance equal to 1. The second pass was a clustering with an aggregation distance equal to 3. Taxonomic assignment of amplicons into operational taxonomic units (OTUs) was produced by Blast+ v2.2.30+ with the Silva 132 Parc databank (Max Planck Institute for Marine Microbiology and Jacobs University, Bremen, Germany). The following specific filters were applied to the pipeline: the last ten bases of reads R1 and R2 were removed (lower quality preventing good read pairing); amplicons shorter than 350 nucleotides or longer than 500 nucleotides were removed; and OTUs with abundance lower than 0·005 per cent of the whole data set abundance were removed. For additional quality control of the sequencing data, all sequence files were assessed with the software FastQC version 0.11.8 (Babraham Bioinformatics, Cambridge, UK). FastQC grades base sequence quality on a quality score. To assess whether the richness of microbiota was captured adequately by metagenomic sequencing, a rarefaction analysis was performed. To ensure a low background signal from bacterial contamination of reagents and consumables, two types of negative control consisting of molecular grade water were added to an empty tube separately at the DNA extraction step and at the PCR steps, and amplified and sequenced at the same time as the DNA extracted from the blood samples.

Statistical analysis

Continuous variables were assessed for normality using the Shapiro–Wilk test. Values for the number of 16S rDNA copies per μl whole blood were log transformed to obtain normality. Median (i.q.r.) values were calculated for continuous variables. A linear mixed-effects model was used for statistical testing of the number of 16S rDNA copies per μl whole blood, with the day of surgery as a fixed effect and the individual patients as random effects. α-Diversity at the OTU level was calculated to investigate the diversity of taxa in each sample. The observed number of OTUs demonstrated α-diversity in terms of richness (number of taxa present in the sample). The Shannon index was used to assess the α-diversity regarding the evenness of taxa in the samples. The α-diversity between time points was compared using the paired Student's *t* test. Relative abundances of bacterial phyla were compared by means of the Wilcoxon signed-rank test. Samples obtained at different time points were defined as distinct ecological communities in order to calculate β-diversity at the OTU level using unweighted UniFrac methodology, which incorporates phylogenetic information[33]. Multidimensional scaling (MDS) ordination was performed to visualize the global level of divergence between individual bacterial profiles at the different sampling times, whereas permutational multivariable ANOVA (PERMANOVA) was used to compare the different days of sampling.

Fig. 2 Mean relative abundance of bacterial phyla in whole blood of all patients stratified by day of sampling



POD, postoperative day.

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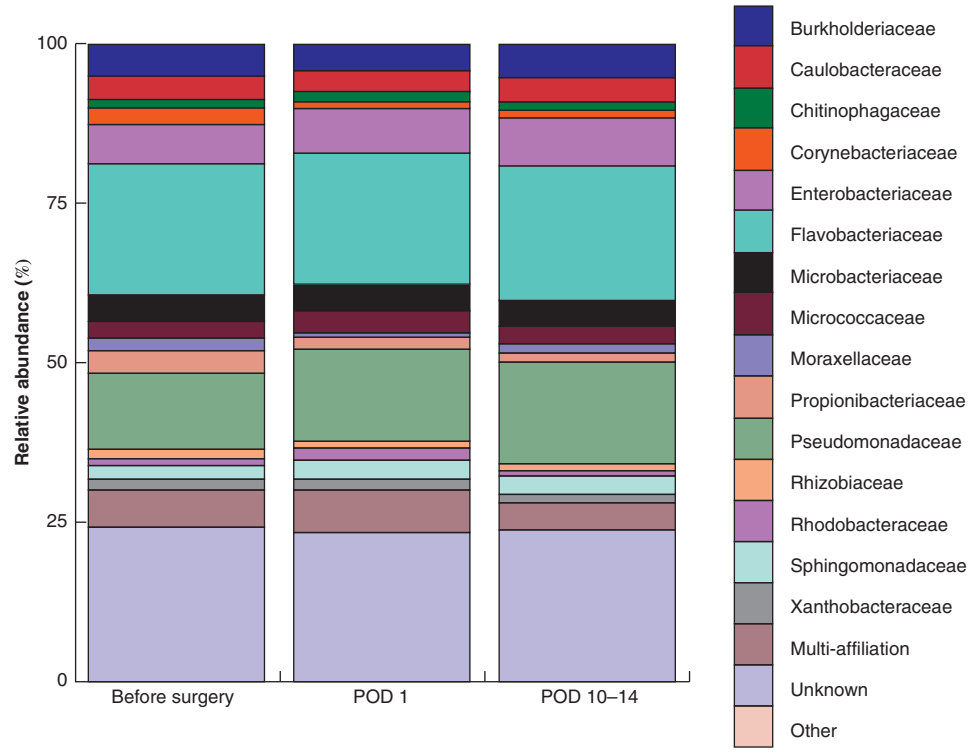
Fig. 3 Mean relative abundance of bacterial families in whole blood of all patients stratified by day of sampling



POD, postoperative day.

Differences in relative abundance of OTUs between time points were analysed using the linear discriminant analysis (LDA) effect size (LEfSe) algorithm with the per-sample normalization of sum values option[34]. LEfSe was run using an α value of 0·05 for the factorial Kruskal–Wallis test among classes, and a threshold of 2·0 for the logarithmic LDA score for discriminative features. As the LEfSe algorithm does not correct for multiple hypothesis testing, the Benjamini–Hochberg false discovery rate procedure was applied. Taxa with a significant difference between sampling times ($P < 0·050$) and a false discovery rate q-value lower than 0·2 were considered the main taxa differentiating between time points.

Statistical analyses were done using R version 3.5.1 (R Core Team, Boston, Massachusetts, USA) including the packages PhyloSeq version 1.24.2 and LMERtest version 3.0-1, unless specified otherwise.

Results

Between January and July 2016, 42 consecutive patients met the inclusion criteria, of whom 12 were later excluded. Two of the excluded patients received blood transfusions during primary surgery, one had benign disease, five had anastomotic leakage, one had postoperative bowel obstruction, two had a postoperative wound infection diagnosed on day 10 after surgery, and one patient was diagnosed with chronic lymphatic leukaemia during the postoperative period. All 30 patients included in the study underwent minimally invasive colonic cancer surgery within an ERAS regimen (*Table 1*). Median duration of hospital stay was 2 (i.q.r. 1–3) days

16S rDNA

In a linear mixed-effects model, the number of 16S rDNA copies per μl whole blood increased by a factor of 1·12 (95 per cent c.i. 1·02 to 1·25) on POD 1 compared with the day before surgery ($P = 0·025$) (*Fig. 1a*). There was no significant difference between POD 10–14 and either POD 1 or the day before surgery.

Microbial diversity

The mean number of raw read pairs per sample was approximately 45 000, of which the mean number of read pairs

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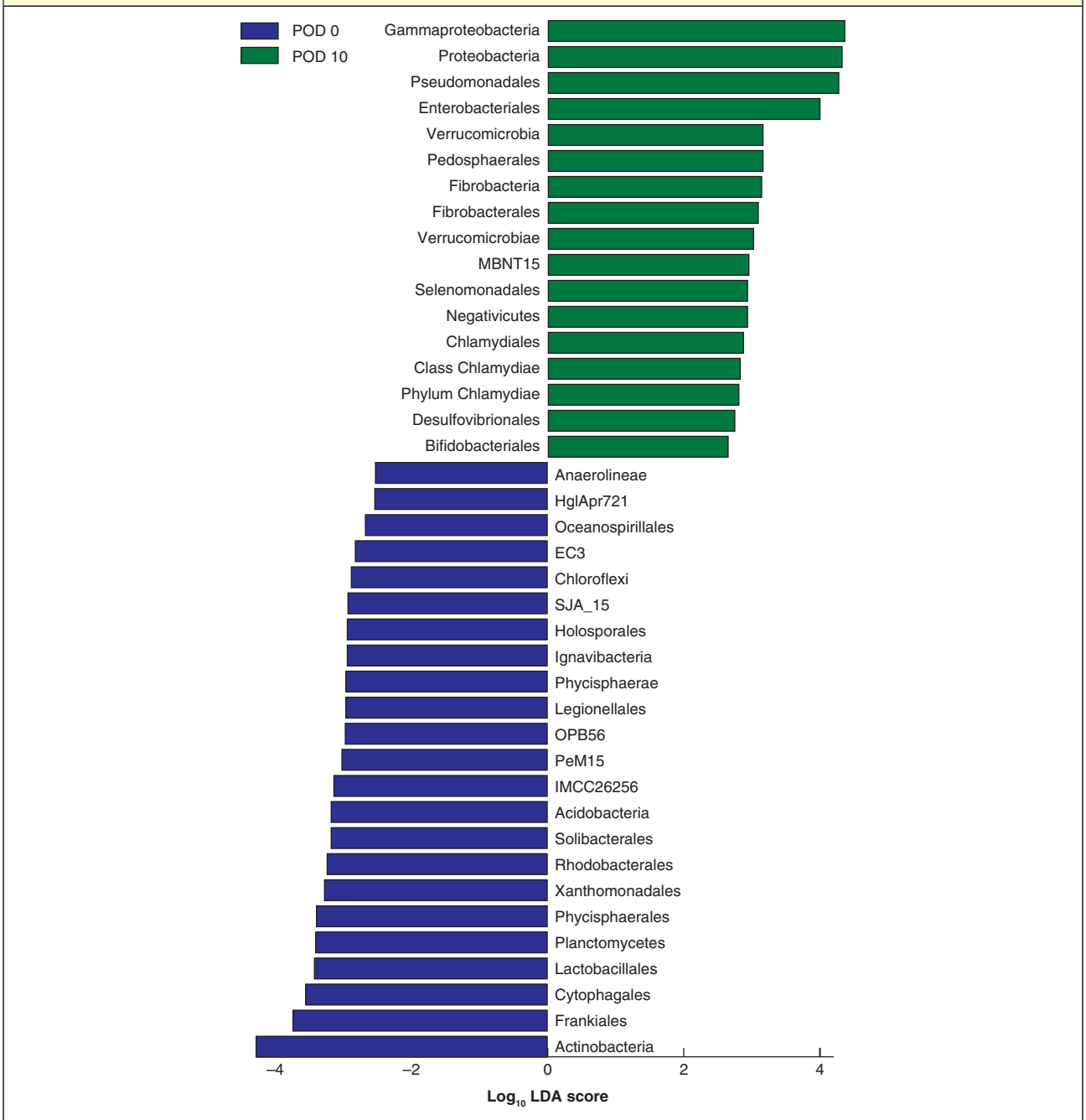
Fig. 4 Linear discriminant analyses effect size algorithm comparing relative abundance at taxonomic levels of phylum, class and order



Bacteria relatively enriched on the day before surgery compared with postoperative day (POD) 10–14 are labelled POD 0; those enriched on POD 10–14 compared with the day before surgery are labelled POD 10. LDA, linear discriminant analysis.

classified in OTUs was approximately 30 000 per sample. No sequences were excluded after FastQC quality control. The rarefaction analysis curves suggested that the sample diversity was captured as expected given that the plateau had been reached (*Fig. S1*, supporting information). Both α-diversity (*Fig. S2a*, supporting information) and β-diversity (*Fig. S2b*, supporting information) analyses showed a clear separation between both negative controls

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and blood samples, suggesting that bacterial contamination was well contained in the pipeline and had a negligible impact on the taxonomic profiles of the samples in the present study.

OTU richness, measured as the mean number of OTUs observed, decreased in the perioperative period and was lower on POD 1 than on the day before surgery (mean difference 8·933, 95 per cent c.i. 0·015 to 17·852; $P = 0·049$), and on POD 10–14 compared with both the day before surgery (mean difference 24·600, 17·083 to 32·117; $P < 0·001$) and POD 1 (mean difference 15·667, 7·447 to 23·886; $P < 0·001$) (*Fig. 1b*). Measurement of α-diversity by the Shannon index showed that the diversity was reduced on POD 10–14 compared with the day before surgery (mean difference 0·097, −0·002 to 0·196; $P = 0·055$) (*Fig. 1c*). MDS analyses of OTU β-diversity calculated using unweighted UniFrac methodology showed separation of the clusters representing the day before surgery and POD 10–14 ($P = 0·001$, PERMANOVA) although there was some overlap (*Fig. 1d*).

Alterations in relative abundance

The relative abundance of bacterial phyla in whole blood from all patients is shown in *Fig. 2*. More than 95 per cent of all sequences in the overall population on all sampling days belonged to the four phyla Proteobacteria (47·6 per cent), Bacteroidetes (28·5 per cent), Actinobacteria (16·4 per cent) and Firmicutes (4·4 per cent). The relative abundances of these phyla were relatively stable across the time points. However, the relative abundance of Actinobacteria decreased between the day before surgery and POD 1 (from a mean of 19·4 to 15·5 per cent; $P = 0·045$) and POD 10–14 (from 19·4 to 14·4 per cent; $P = 0·040$). At the family level, the relative abundances of the 15 most abundant families were relatively stable between the sampling times (*Fig. 3*).

With richness and diversity measures showing the greatest response between the day before surgery and POD 10–14, it was decided to undertake LEfSe analysis to identify which differences in the relative abundance of bacteria at different taxonomic levels may drive the decrease in richness and altered β-diversity. LEfSe analysis at all taxonomic levels down to species level revealed numerous relatively enriched taxa; 96 taxa were relatively enriched on the day before surgery and 63 on POD 10–14 (*Table S1*, supporting information). Among these, the species *Fusobacterium nucleatum* was relatively enriched on POD 10–14. *F. nucleatum* was not present in any preoperative samples but was detected in five patients on POD 10–14, representing between 0·003 and 2 per cent of all reads in these patients. For better readability, a restricted LEfSe analysis of the taxonomic levels of phylum, class and order is provided in *Fig. 4*. Bacteria relatively enriched before surgery were of the phyla Planctomycetes and Chloroflexi; of the classes Actinobacteria, Acidobacteria, Phycisphaerae, Ignavibacteria and Anaerolineae; and of the orders Frankiales, Cytophagales, Lactobacillales, Xanthomonadales, Rhodobacterales, Solibacterales, IMCC26256 (of class Acidimicrobiia), PeM15 (of class Actinobacteria), OPB56 (of class Ignavibacteria), Legionellales, Phycisphaerales, Holosporales, SJA_15 (of class Anaerolineae), EC3 (of class Gammaproteobacteria), Oceanospirillales and HglApr721 (of class Gammaproteobacteria). Bacteria relatively enriched on postoperative day 10–14 were of the phylum Proteobacteria, Verrucomicrobia and Chlamydiae; of the classes Gammaproteobacteria, Fibrobacteria, Verrucomicrobiae, Negativicutes and Chlamydiae; and of the orders Pseudomonadales, Enterobacteriales, Pedosphaerales, Fibrobacterales, MBNT15 (of class Deltaproteobacteria), Selenomonadales, Chlamydiales, Desulfovibrionales and Bifidobacteriales.

Discussion

These findings of this study suggest that blood of patients with colonic cancer harbours bacterial 16S rDNA from numerous taxa forming a rich microbiota, which is augmented during the immediate postoperative period. The results also suggest that the blood microbiota becomes less diverse with relative enrichment of specific bacteria after operation.

Blood microbiota has not been described previously in patients undergoing surgery for colonic cancer. In a surgical setting, blood microbiota has been studied in an RCT[35] of the effect of perioperative synbiotics in patients undergoing oesophagectomy for oesophageal cancer. In that study, a higher proportion of patients had detectable bacteraemia defined by the presence of any of 11 prespecified groups or species of bacteria in a reverse transcriptase–qPCR assay after surgery compared with before operation. The use of synbiotics reduced the number of patients with postoperative bacteraemia and neutrophil counts, suggesting a beneficial effect on the surgical stress response[35]. Using an unrestricted approach with universal bacterial primers, the present findings suggest that bacterial 16S rDNA is present in all patients with colonic cancer and that its concentration is increased after operation. The role of the gut as a barrier to bacterial translocation has been demonstrated in several conditions, such as inflammatory bowel disease, sepsis and intestinal

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obstruction; such patients had higher concentrations of bacterial DNA in the blood than patients with inactive disease and healthy subjects[36].

The use of a 16S rDNA sequence-based technique in the present study allowed a comprehensive quantitative and qualitative description of the microbiota present in the blood of patients at different time points during the course of potentially curative colorectal cancer surgery. The standardized workflow in this study has been validated previously and the universal primers used provide high sensitivity (targeting 95 per cent of all bacterial sequences in the Ribosomal Database Project) and 100 per cent specificity (no mitochondrial, archaea or eukaryotic DNA targeted). Furthermore, the workflow has been optimized to ensure a very low overall background signal from bacterial contaminants present in reagents and consumables[19,23,24]. Although more OTUs were identified in the DNA extraction negative control than the PCR negative control, suggesting some level of contamination from the DNA extraction step, the overall level of OTUs in the negative controls was clearly lower than in blood samples, and they formed separate clusters in MDS.

The methods applied here do not provide any information on the direct source of the microbiota identified. Hence, the gut as a source and bacterial translocation as mechanism remains a hypothesis in this study. Obtaining the blood by peripheral transcutaneous sampling carries the risk of contamination from skin commensals. Skin commensals, such as the bacterial families Staphylococcaceae and Propionibacteriaceae were observed, but these accounted for only about 3 per cent of the total abundance at this taxonomic level. Nor do the methods applied provide information on the viability of the bacteria from which the 16S rDNA was identified. It could merely represent degraded components of phagocytosed bacteria. Although human blood has traditionally been considered a sterile compartment, studies have demonstrated that blood from healthy donors harbours both viable bacteria and rich microbiota. Bacteraemia may indeed be an everyday phenomenon occurring during tooth brushing[37].

The exploratory nature of the present study limits the ability to draw firm conclusions. A non-significant reduction in α-diversity measured by the Shannon index was observed on POD 10–14 compared with the day before surgery ($P = 0{\cdot}055$), but this result may have been prone to a type II error due to the small population size. Furthermore, the colonic microbiota varies between different parts of the colon in patients with colorectal cancer[38]. The resections in this study were performed on different segments of the colon, which may have led to data heterogeneity. The differences in blood microbiota measured at different time points may have been influenced by the antibiotics given at the induction of anaesthesia. Previous studies have shown decreased α-diversity of gut microbiota after a single dose of oral antibiotics[39]. Furthermore, preoperative enemas may have caused perturbations in the microbiota before surgery and contributed to variation. However, prophylactic antibiotics and enemas are part of the standard surgical care in the authors' department, and the microbiotic phenotype of the peripheral blood of patients with colonic cancer is a product of the combined surgical pathway, including anaesthetics and antibiotics. The type and doses of antibiotics were similar in all patients.

The findings of this exploratory study raise new hypotheses about possible interactions between colorectal cancer, colorectal surgery and blood microbiota. Whether malignant disease alters the blood microbiota, or the present findings reflect the effect of gastrointestinal surgery, could be tested by including a control group of patients undergoing colonic resection for benign indications. In addition, future trials not excluding patients with postoperative complications would be of interest to examine the impact of these on the blood microbiota profile, and possibly validate the method as a biomarker for complications such as subclinical anastomotic leakage. Finally, incorporating long-term oncological outcomes into prospective trials would provide evidence of possible interactions between prognosis and the blood microbiota.

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