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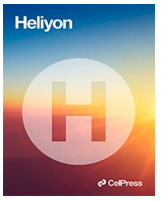

Research article

The baseline fecal microbiome differs in patients with and without anastomotic leakage after colorectal cancer surgery

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A B S T R A C T

Background: Anastomotic leakage (AL) is a severe complication of colorectal surgery. The risk of AL is affected by both surgery and patient factors. Gut microbiomes can be generated from the residual material from the fecal immunochemical test (FIT). We, therefore, examined if AL after colorectal cancer surgery could be associated with specific baseline microbiomes in the FIT screening sampling tubes collected weeks before surgery.
Methods: Samples from patients participating in the Danish colorectal cancer screening program were biobanked from 2016 to 2018, and samples from patients who had surgery for screening-detected cancer were included. They were matched with patients without AL in a 1:2 ratio based on age, sex, location of anastomosis (colonic/rectal), ASA classification, and smoking habits. Bacterial DNA was extracted from the sampling tubes, and the fecal microbiomes were analyzed with targeted 16S ribosomal RNA third-generation sequencing.
Results: 18 patients who developed AL after surgery were matched with 36 without AL. The alpha diversity was lower in the AL group ($p = 0.035$), and the AL group separated from the Controls in the PCoA plot ($p < 0.001$). This was due to the patients undergoing rectal resections, with significant differences in alpha- and beta diversity ($p = 0.025$ and $p = 0.002$, respectively). The prevalence of bacteria with the potential to produce collagenase was higher in patients who developed AL (odds ratio 1.29 (95% CI 1.28–1.30), $p < 0.001$).
Conclusions: We found differences in the baseline microbiome profile associated with subsequent development of AL after surgery for screening-detected rectal cancer.

1. Introduction

Colorectal cancer (CRC) is among the most common malignancies, and to reduce morbidity and mortality, a fecal immunochemical test (FIT) based screening program was introduced in Denmark in 2014 [1]. This has led to screening CRCs being detected at lower stages, and most screening-detected CRCs are resectable at the time of diagnosis [2]. In most patients, it is possible to re-establish gut continuity with an anastomosis. Postoperative complications of colorectal surgery are, however, still frequent, affecting up to one-third

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of all patients [3]. Anastomotic leakage (AL) is one of the most severe postoperative complications, as it usually implies reoperation with resection of the leakage site and the formation of a stoma [4]. Patients with little or no clinical affection can be handled conservatively, e.g., using percutaneous drainage or endoscopic treatment [5]. Regardless of the severity, in general, AL entails increased costs, decreased quality of life, increased risk of cancer recurrence, and increased mortality [6–8].

Multiple risk factors predisposing to AL have been identified and can be divided into modifiable or non-modifiable factors. Non-modifiable risk factors for AL include sex (men have a higher risk than women) and increasing age [9,10]. The modifiable risk factors of colorectal AL include hyperglycemia, anemia, blood loss during surgery, blood transfusion, intraoperative events, contamination, and conversion from laparoscopic to open surgery, as well as the timing (elective/acute) and duration of surgery [11]. Other modifiable factors are related to lifestyle (e.g., smoking habits, consumption of alcohol, being overweight) [12,13] or chronic illnesses, including the use of immunosuppressive drugs [14]. The risk of AL varies from 2 to 15 % depending on the anatomical site of the anastomosis. Anastomoses to the right side of the colon have the lowest risk, and anastomoses to the rectum imply the highest risk of AL [14,15].

Several modifiable and non-modifiable factors also affect the host's baseline microbiome [16]. For instance, the composition of the gut baseline microbiome varies with the anatomical location in the intestines, and it is also affected by the host's immune status [17]. Furthermore, the perioperative gut microbiome is affected by stressors (e.g., mechanical bowel cleansing) [18], during (e.g., surgical stress response or antibiotics) [19], and after surgery (e.g., antibiotics, proton pump inhibitors, other medications, and change of diet) [16]. These facilitate dysbiosis (decrease in bacterial diversity and overrepresentation of harmful species) [20], which might contribute to AL development [21]. Fortunately, the preoperative changes in the microbiome can be counteracted with dietary prehabilitation [22].

Experimental studies have shown a causal linkage between the gut microbiome and the development of AL after colorectal surgery [23], including certain strains of bacteria (e.g., *Enterococcus faecalis* or *Pseudomonas aeruginosa*) that apparently work through activation of collagenases adjacent to the colonic anastomosis [24,25].

This study examined whether the baseline fecal microbiome of patients undergoing CRC resection for screening-detected cancer differs between patients suffering from postoperative AL and patients without AL. Further, we wanted to determine whether the prevalence of bacteria with known capacity for collagenase production was higher in patients with postoperative AL.

2. Methods

This paper was outlined according to the "Strengthening The Organization and Reporting of Microbiome Studies" (STORMS) checklist (Supplementary Material) [26].

The study was approved by the Committee of Research Ethics in Region Zealand (EMN-2021-04114), which allowed us to conduct it without obtaining informed consent from the patients. Region Zealand also approved the collection and storage of data (REG-056-2021 and REG-46-2016).

2.1. Study participants

This retrospective case-control study involved screening-detected CRC patients in Region Zealand, Denmark, which has about 800,000 inhabitants. Patients underwent colonic or rectal resection with anastomosis formation at two colorectal centers. FIT screening samples were stored in the Region Zealand CRC screening biobank from 2016 to 2018. The Danish Colorectal Cancer Group database provided the majority of the clinical data for the study. Still, we supplemented it by extracting a few data from the patient records (level of anastomosis for sigmoid/rectal resections, use of rectal washout, and the durations from colonoscopy to surgery). Long-term prescription medication data affecting the gut microbiota (e.g., proton pump inhibitors (PPI), non-steroidal anti-inflammatory drugs (NSAID), opioids, antibiotics, steroids, chemotherapy) were gathered from admission records. However, general practitioners often prescribe information on short-term antibiotic courses and other medications (e.g., PPI, NSAID) that are not consistently printed in hospital records. Thus, obtaining complete and accurate antibiotic usage data for the three months preceding sample collection and hospital admission was impossible.

2.2. Definition and diagnosis of anastomotic leakage

In this study, the definition follows that of the International Study Group of Rectal Cancer, which defines AL as "a defect of the intestinal wall integrity at the colorectal or colo-anal anastomotic site leading to a communication between the intra- and extraluminal compartments" [27]. This guideline also includes a grading system based on the level of intervention required to handle the AL. Even though this definition does not mention ileocolic or colo-colic anastomoses, it is widely used to classify leakage to these types of anastomoses as well, both in the surgical literature and in the database of the Danish Colorectal Cancer Group, including data from all patients with colorectal cancer in Denmark. The database has >95 % patient and data completeness [28].

Anastomotic leakage was diagnosed by combining clinical symptoms with paraclinical tests, including radiological confirmation of AL and perioperative confirmation.

2.3. Matching

Patients having surgery with the formation of an anastomosis in Region Zealand were identified with data from the Danish

Colorectal Cancer Group's database, and the patients referred through the CRC screening program were selected. The patients suffering postoperative AL (AL group) were identified and matched with similar patients without AL (Control group) in a 1:2-ratio, based on age, sex, type of resection (concerning the location of the anastomosis, e.g., colonic/rectal), smoker habits, and through the American Society of Anesthesiologists (ASA) classification. This was done to minimize the influence of important known confounders for AL.

2.4. Primary and secondary outcomes

The study's primary outcome was identifying and describing differences in the baseline microbiome in the fecal screening samples between the AL and Control groups.

The study's secondary outcomes were subset analyses of the two groups, e.g., divided into colonic vs. rectal resections (whether the anastomosis involved the rectum) and analysis for bacteria with a known potential to produce collagenases.

2.5. Sample collection

Invitations for participation in the national Danish colorectal cancer FIT-based screening program, a collection tube, and instructions for sample collection are mailed to citizens 50–74 years old bi-annually [1]. The written instructions include a cartoon depicting how to collect the FIT sample from the surface of the fecal matter. After collection, the samples are mailed to the regional testing center within one to three days of transit at ambient temperature (Supplementary Fig. S1). The cutoff for the FIT test is $\geq$ 100 μg Hb/L (equivalent to 20 mg Hb/g feces) [1]. The sampling tube (OC-Auto Sampling Bottle 3, Eiken Chemical Co., Ltd., Tokyo, Japan) contained 2 ml of HEPES buffer (N-2-hydroxyethylpiperazine-N′-2-ethane-sulforic acid) and collected approximately 10 mg of feces per sample. Within two weeks of being found FIT positive, the patients are scheduled for a screening colonoscopy. If a CRC is found, the patients are planned for surgery (Fig. 1). From 2016 to 2018, all FIT-positive samples were collected and stored in a biobank at −80 °C until analysis.

2.6. DNA extraction, microbiome preparation, and sequencing

For a detailed description, see Supplementary Table S1. To extract the bacterial DNA, the frozen sampling tubes were thawed, the contents mixed, and bacterial DNA extracted using the AllPrep PowerFecal DNA/RNA Kit (Qiagen, Hilden, Germany). The 16S rRNA V1-V9 regions were amplified via polymerase chain reaction, and sequencing was performed on the MinION Mk1C from Oxford Nanopore Technologies.

2.7. Software and data processing

MinKNOW version 5.7.5 was used for base calling with Dorado version 7.1.4. Reads not meeting quality criteria were discarded. Taxonomy was classified according to the Basic Local Alignment Search Tool from the National Center of Biotechnology Information, and statistical analysis was conducted using RStudio version 2023.09.1 with phyloseq version 1.44.0 and microbiome version 1.22.0 packages. For full details, see Supplementary Table S2.

2.8. Statistical calculations

Rarefaction curves were done for all samples to assess the quality, and normalization was performed (number of reads of each species/number of total reads per sample). Only full-length sequences were included in the analyses. Alpha diversity metrics of the AL and Control groups were calculated using the Shannon diversity index. We used the Shannon diversity index because it balances species richness and evenness, capturing overall diversity more comprehensively than the Simpson index (which emphasizes

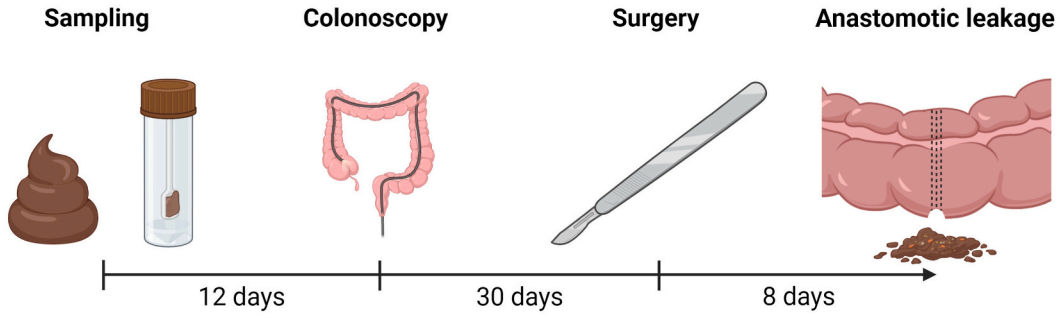

Fig. 1. Timeline from fecal screening sample collection to colonoscopy, surgery, and subsequent anastomotic leakage for patients of the AL group. The number of days is a medium value for the combined cohort.

dominance) or the Chao1 index (which focuses solely on richness). Differences between the alpha diversity of the groups were calculated using the Wilcoxon Rank-Sum test. The beta diversity between the compositions of the two groups was calculated with Canberra distance and visualized in a Principal Coordinate Analysis (PCoA) plot. We used the Canberra distance for beta diversity analysis due to its sensitivity to rare taxa, which may be crucial in detecting subtle shifts in low-abundance microbial species associated with disease or surgery outcomes. Differences between the beta diversity of the groups were calculated using the permutational analysis of variance (PERMANOVA) test. The relative abundances of the samples were visualized as stacked bar plots, and the proportions were calculated with the Wilcoxon Rank-Sum test (detection = 0.5 %, and prevalence = 50 %), with the Benjamini-Hochberg correction to adjust for the false discovery rate. Because the risk for rectal AL differs from that of the rest of the colon, this was analyzed by subsetting the dataset into colonic or rectal resection. Furthermore, the prevalence of bacteria that could potentially produce collagenase (complete list presented in Supplementary Table S3) in patients with and without AL was investigated in both subgroups. All values were presented as median with interquartile range (IQR). Fisher's exact was used to calculate the association of two categorical variables. We chose to show the analyses relevant to the planned outcomes. P-values <0.05 were considered statistically significant.

3. Results

3.1. Cohort description

Patients having surgery for CRC in Region Zealand in 2016–2018 were identified through the Danish Colorectal Cancer Group database. We included the patients having surgery with the formation of an anastomosis and referred through the Danish CRC screening program (Fig. 2). It was possible to retrieve FIT samples from the biobank for 18 patients out of the 31 patients who developed AL after surgery for screening-detected cancer. According to the surgical records, no apparent reasons for AL (tissue ischemia, traction of the anastomosis, or other technical failures) were present. The baseline characteristics are shown in Table 1. Although there were minor differences between the groups regarding age, body mass index, smoker status, alcohol consumption, performance status, and ASA classification, none reached significance in the statistical tests. Three patients in both the AL and the

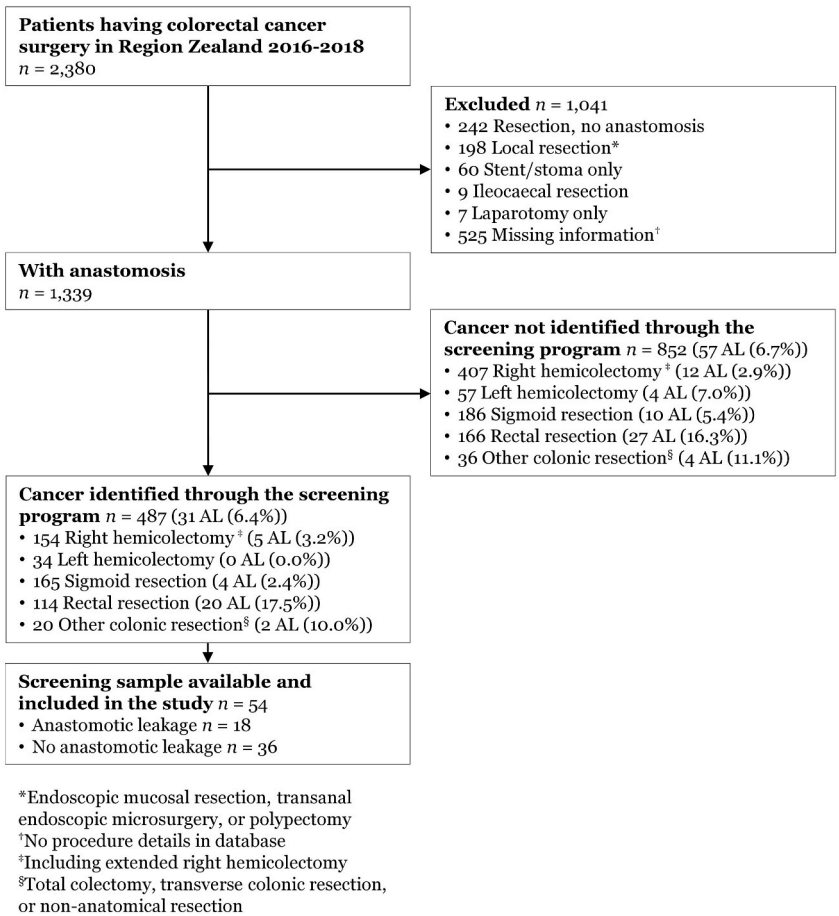

Fig. 2. Trial profile of all patients in Region Zealand registered in the Danish Colorectal Cancer Group database from 2016 to 2018.

Table 1
Patient characteristics.

	Anastomotic leakage (N = 18)	No anastomotic leakage (N = 36)
Age (years)	64 (61.5–71.8)	66 (60.8–71.0)
Sex		
Male	13 (72.2)	26 (72.2)
Female	5 (27.8)	10 (27.8)
BMI (kg/m^2)	28.1 (26.5–32.0)	27.1 (24.5–30.0)
Smoking		
Never smoker	5 (27.8)	14 (38.9)
Previous smoker	9 (50.0)	12 (33.3)
Current smoker	4 (22.2)	10 (27.8)
Alcohol consumption		
No consumption	1 (5.6)	7 (19.4)
Within recommendations	13 (72.2)	25 (69.4)
Above recommendations	4 (22.2)	4 (11.1)
ASA classification		
I	8 (44.4)	19 (52.8)
II	9 (50.0)	16 (44.4)
III	1 (5.6)	1 (2.8)
WHO performance status		
0	0 (0)	0 (0)
1	17 (94.4)	35 (97.2)
2	1 (5.6)	0 (0)
3	0 (0)	1 (2.8)
Tumor location		
Colon	4 (22.2)	11 (30.6)
Rectum	14 (77.8)	25 (69.4)
Anastomotic leakage, grade		
A	1 (5.6)	NA
B	9 (50.0)	NA
C	8 (44.4)	NA
Time (days)		
Faecal sample to colonoscopy	14 (9–18)	10 (8–12)
Colonoscopy to MDT decision	11 (8–13)	10 (8–14)
MDT decision to surgery	20 (14–26)	15 (8–24)
Surgery to anastomotic leakage	8 (5–11)	NA
Fecal sample to surgery[a]	48 (40–55)	42 (32–49)
Fecal sample to anastomotic leakage	57 (46–71)	NA

Data are median (interquartile range) or number (percentage).
BMI, body mass index; ASA, American Society of Anesthesiologists; WHO, World Health Organisation; MDT, multidisciplinary team; NA, not applicable.
[a] There was an outlier in each group. In the AL group, one patient waited 281 days from the fecal sample to surgery because of neoadjuvant chemotherapy. In the Control group, one patient waited 322 days from the fecal sample to surgery because of neoadjuvant radiation therapy.

Control group had daily use of PPI. In the AL group, one used an antimetabolite, one used NSAID, and one used steroids daily. Similarly, only a few differences existed between the groups concerning the surgical procedures (Table 2). There was one T4-staged tumor in the AL group, whereas this group had no T1-staged tumors. Three operations of the AL group were converted from a laparoscopic approach to laparotomy. The fraction of robotic-assisted operations was higher in the AL group, but the fraction of the transanal total mesocolic excision (TaTME) procedure was lower.

3.2. Sequencing depth

The median number of sequenced reads was 193,351 (range 56,613–366,947) among the AL patients and 86,915 reads (range 5,344–271,278) for patients in the Control group (Supplementary Fig. S2). The DNA sequences' read lengths were 1,436 nucleotides (IQR 1,424-1,441), and the Phred score was 12.6 (IQR 12.1–13.4). The accuracy of BLAST identification was 94.7 % (IQR 91.6%–96.7 %). The rarefaction curves are shown in Supplementary Fig. S3.

3.3. AL vs. Control group

The median alpha diversity was lower in the AL group than in the Control group (3.17 (IQR 2.82–3.50) vs. 3.56 (IQR 3.11–3.81); $p = 0.035$; Supplementary Fig. S4). The two groups also significantly differed in the PCoA plot and PERMANOVA analysis ($p < 0.001$; Supplementary Fig. S5). The relative abundances showed that most of the bacteria in the two groups belonged to the phylum *Bacillota* (formerly known as *Firmicutes*), see Fig. 3A and B. The AL group had fewer *Bacteroidota* than the Control group ($p < 0.01$), which is also reflected in the lower levels of the taxonomy (class *Bacteroidia* ($p < 0.05$) and order *Bacteroidales* ($p < 0.05$)). Even though *Pseudomonadota* seemed more abundant in the AL group, the difference was insignificant. On the species level, the only significant difference

Table 2
Operative data.

	Anastomotic leakage (N = 18)	No anastomotic leakage (N = 36)
Tumour localisation		
Ascending colon	1 (5.6)	2 (5.6)
Hepatic flexure	1 (5.6)	2 (5.6)
Transverse colon	1 (5.6)	2 (5.6)
Splenic flexure	1 (5.6)	2 (5.6)
Sigmoid colon	0 (0)	3 (8.3)
Rectum	14 (77.8)	25 (69.4)
Clinical T-stadium		
T1	0 (0)	3 (8.3)
T2	9 (50.0)	18 (50.0)
T3	7 (38.9)	13 (36.1)
T4	1 (5.6)	0 (0)
Tx	1 (5.6)	2 (5.6)
Clinical N-stadium		
N0	10 (55.6)	13 (36.1)
N1	2 (11.1)	6 (16.7)
N2	1 (5.6)	7 (19.4)
Nx	5 (27.8)	10 (27.8)
Clinical M-stadium		
M0	16 (88.9)	34 (94.4)
M1	2 (11.1)	2 (5.6)
Surgical procedure		
Right hemicolectomy	1 (5.6)	2 (5.6)
Extended right hemicolectomy	2 (11.1)	4 (11.1)
Other colonic resection	1 (5.6)	2 (5.6)
Rectal resection	14 (77.8)	28 (77.8)
Surgical method/approach		
Laparoscopic	9 (50.0)	18 (50.0)
Robotic-assisted	2 (11.1)	1 (2.8)
TaTME	7 (38.9)	17 (47.2)
Converted to laparotomy	3 (16.7)	0 (0)
Anastomosis		
Stapled	16 (88.9)	32 (88.9)
Hand-sewn	2 (11.1)	4 (11.1)
Sigmoid/Rectal resections[a]	N = 14	N = 28
Loop ileostomy	11 (78.6)	22 (78.6)
Rectal washout[b]	10 (71.4)	22 (78.6)
Level of anastomosis, cm	5.0 (4.0–5.8)	5.0 (4.0–7.0)
Tumor distance from the anal verge, cm		
Clinical	9.5 (8.0–11.0)	10.0 (7.0–11.0)
MRI scan	8.5 (8.0–12.0)	10.0 (7.0–11.0)

Data are median (interquartile range) or number (percentage).
TaTME, transanal total mesocolic excision; MRI, magnetic resonance imaging.
[a] Combined as they all have a circular stapled anastomosis to the rectum.
[b] Before the construction of an anastomosis. There were missing values for four patients in the AL group and three in the non-AL group.

was that *Oscillibacter valericigenes* were less abundant in the AL group ($p < 0.05$).

3.4. Colonic vs. rectal resections

There was no difference in alpha diversity (3.28 (IQR 2.93–3.61) vs. 3.36 (IQR 3.07–3.59); $p = 0.93$) or beta diversity ($p = 0.224$) for the patients having a colonic resection with or without AL. In contrast, the alpha diversity was significantly lower in the patients who developed AL after rectal cancer surgery (3.17 (IQR 2.82–3.42) vs. 3.65 (IQR 3.13–3.87); $p = 0.025$; Fig. 4), as was the beta diversity ($p = 0.002$; Fig. 5). In the microbiomes of patients who had AL following rectal resection, we found a lower relative abundance of the phylum *Bacteroidota* ($p < 0.05$), class *Bacteroidia* ($p < 0.05$), and order *Bacteroidales* ($p < 0.05$). On the species level, *Blautia obeum* was more abundant in the AL group ($p < 0.05$). In contrast, *Oscillibacter valericigenes* was less abundant ($p < 0.05$). We found no differences in the relative abundances in the microbiomes of the patients having colonic resection.

3.5. Bacteria with the potential to produce collagenase

The data was subset to include only reads from bacteria known to have the potential to produce collagenase (Supplementary Table S3). We found no difference in alpha diversity (Supplementary Fig. S6). However, 3.81 % of all reads in the samples from patients who developed AL were from bacteria known to produce collagenases, compared with 2.98 % of all reads in the Control group, giving an odds ratio of 1.29 (95% CI 1.28–1.30, $p < 0.001$). The beta diversity differed overall ($p = 0.014$) and among patients

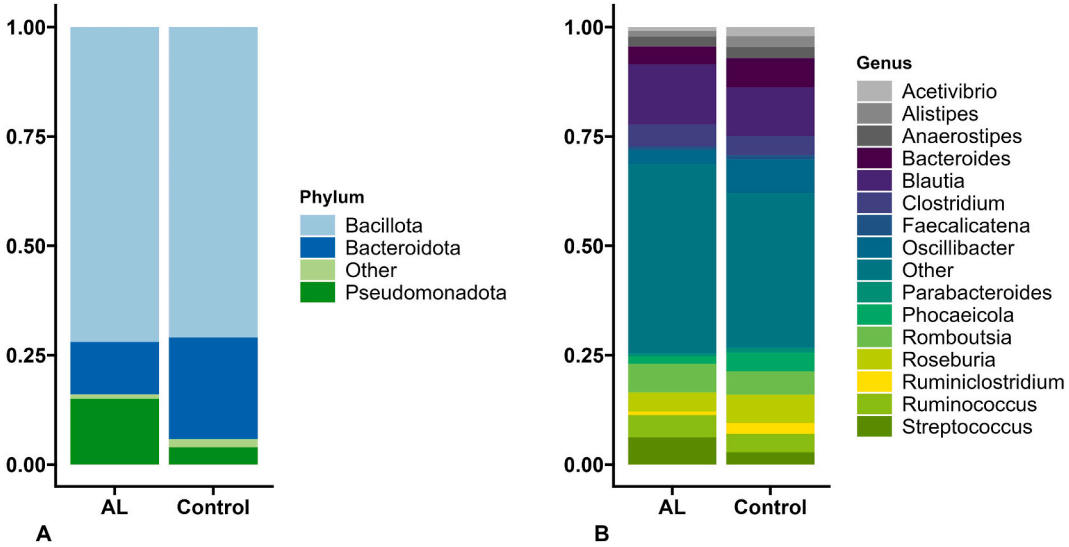

Fig. 3. Relative abundances, AL vs. Control group. The detection rate was set to 0.5 %, the lower limit of the proportion a particular taxonomic group should constitute in a sample to be included in the analysis. The prevalence rate was set to 50 %, which means that the specific taxonomic group should be present in at least 50 % of all samples in the cohort to be included in the analysis. (A) Phylum level. (B) Genus level.

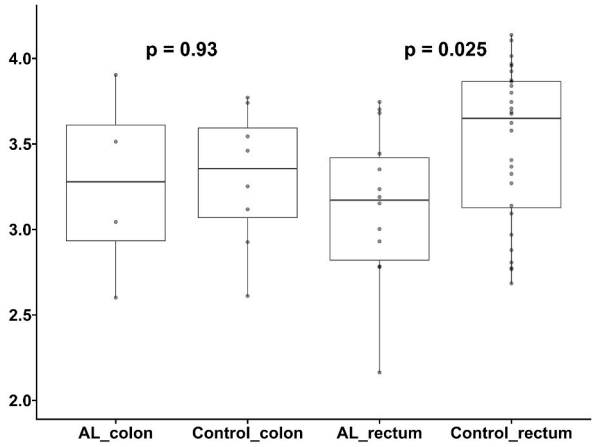

Fig. 4. Alpha diversity (Shannon diversity index), colonic vs. rectal resections. Median values are marked with a horizontal line, and the box designates the 25 % and 75 % quartiles. The difference between the groups is calculated using the Wilcoxon rank-sum test.

undergoing rectal resection (p = 0.01; Supplementary Fig. S7).

3.6. Generalizability of the study

This study solely analyzed patients with screening-detected CRCs. To assess generalizability, we compared CRC incidence and anastomotic leakage risk by anatomical location between screening-detected and non-screening-detected cancers. From 2016 to 2018, approximately one-third of new CRCs in Region Zealand were screening-detected, with a higher proportion of distal tumors. Nonetheless, the risk of anastomotic leakage at each location was similar between screening and non-screening groups (Supplementary Table S4).

4. Discussion

This study demonstrated that microbiome sequencing from bacterial DNA extracted from the residual material in the FIT-screening tubes is possible. We identified several differences in the baseline fecal microbiome profiles between patients with and without AL after colorectal cancer surgery. The sequenced microbiomes reflect long-term gut bacteria composition 1–2 months before surgery in presumably asymptomatic patients. Unlike many microbiome studies focusing on closer-to-surgery composition, our study offers

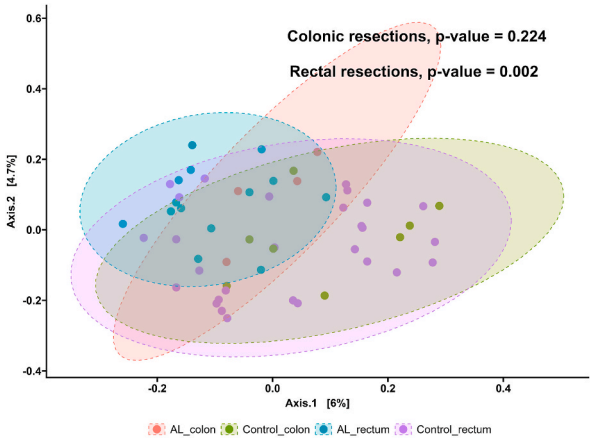

Fig. 5. Principal Coordinates Analysis plot (Canberra distance), colonic vs. rectal resections. The Axis.1 depicts the dimension with the most significant variation (in percent) of the samples and the Axis.2 depicts the dimension of the second-most variation (in percent).

insight into general host-gut bacteria balance using baseline preoperative samples. However, they do not capture perioperative changes like dysbiosis.

Our discovery of lower microbiome alpha diversity in AL patients' screening fecal samples suggests a possible role of gut microbiome composition in AL development or reflecting predisposing host conditions. Recent evidence supports the association between an increased risk for AL and certain microbiomes, demonstrated in a fecal microbiota transplantation model from humans to mice [23]. Characterizing the "normal" gut microbiome is complex due to individual composition variations [29]. Proposed enterotypes, reflecting bacterial abundance from the *Bacteroides*, *Prevotella*, or *Ruminococcus* genera, highlight species composition similarities [30]. No significant differences in the relative abundance of these bacteria were found in our study.

Prior investigations on fecal samples from colorectal cancer surgery patients exhibit variation in the sample material, analytical techniques, and timing of collection. One study compared fecal samples collected after surgery and sequenced on the Illumina platform, revealing over 60 % *Enterococcus* in both AL and control groups [31]. In contrast, our study identified only a minute fraction of *Enterococcus*. Another study analyzed pre-surgery fecal samples, though the duration from collection to surgery was undisclosed [32]. The study showed that two species (*Acinetobacter lwoffii* et *johnsonii*) were present only among AL patients. Although we detected both species in AL and Control patients, the quantities were insignificant between the groups. Studies of the mucus microbiomes of end-to-end ("donut") tissue from stapled colorectal anastomoses showed that *Blautia obeum* was associated with the later occurrence of AL compared with a control group [33]. Our results align with this finding, as *Blautia obeum* was abundant in the baseline microbiomes in the AL group. *Oscillibacter valericigenes* was abundant among the patients in the Control group. This bacteria ferments dietary fibers in the colon, thus producing valeric acid [34], one of the short-chain fatty acids that are the colonocytes' primary energy source. Previous research identified higher *Pseudomonata* (formerly *Proteobacteria*) levels in AL patient samples, including fecal [32] and mucosal specimens [35]. While our findings didn't reach significance, we noted increased *Pseudomonata* presence in the AL group, which may contribute to dysbiosis and colonocyte dysfunction [36]. Altogether, our results support the hypothesis that patients with AL after colorectal surgery have specific patterns of the compositions of the baseline fecal microbiome.

We observed a higher prevalence of bacteria capable of producing collagenase in samples from AL patients, particularly after rectal resections, suggesting elevated collagenase activity. However, detecting messenger RNA or actual collagenase activity was not possible in our study. Only two other clinical studies [37,38] of colorectal AL have examined the association between bacteria with the potential to produce collagenase and AL. One study was a case series in which bacterial cultures from four different patients with AL showed the presence of collagenolytic strains of *Enterococcus faecalis*, *Serratia marcescens*, *Stenotrophomonas malthophilia*, *Proteus mirabilis*, or *Pseudomonas aeruginosa* [37]. In another case series of rectal resection patients, it was shown that a strain of *Enterococcus faecalis* from lavage fluid of an AL had both collagenolytic activity and the capability to activate matrix metalloproteinase-9 (MMP-9) [38]. It had previously been shown that MMP-9 levels are increased in the intraperitoneal fluid of AL patients, although information on the presence of bacteria in this fluid was not provided [39]. In a recent systematic review, we outlined several studies investigating the microbiology of AL patients after colorectal surgery [40]. Most studies identified bacteria with this potential to produce collagenases in feces, mucosa, resected specimens, or drain fluid. However, evidence was lacking to support enzymatic collagenase activity as a potential pathogenic mechanism.

The CRC screening program participants are instructed not to collect the samples while having signs of acute gastrointestinal diseases. They are, therefore, presumed to be "asymptomatic" at the time of sample collection. Many of the bacteria with higher abundance in the AL group are low pathogenic/opportunistic or, if pathogenic, found in very low abundances. This may suggest a different baseline immune function in the patients of the AL group, resembling the observations seen in COVID-19 patients, in which rectal swabs showed an increase in the phylum *Proteobacteria* [41]. However, no test allows us to evaluate patients' immune status. AL incidence at various anastomotic sites was comparable for screening-detected and non-screening cancers, suggesting that our findings could be generalized beyond screening patients. Colorectal cancer patients' microbiota differs from other patients' [42], cautioning

generalization to different populations and benign conditions.

Our study has several strengths. First, it confirmed the feasibility of third-generation sequencing of the complete 16S rRNA gene from fecal sample tubes used in CRC screening programs globally [43]. Second, it aimed to minimize confounding by matching patients across five variables. Although it was impossible to achieve identical accuracy in matching all five parameters, the impact of these minor differences is debatable. The median difference in age between the two groups was two years (with the Control group having the oldest patients). This contradicts the assumption that the risk of AL increases with age. The same goes for smoker status since the Control group had a slightly larger percentage of current smokers. As for the ASA classification, slightly more patients in the AL group were classified as class II or III compared to the Control group. Third, it identified significant differences between groups, backing the notion that the baseline gut microbiome may play a role in colorectal AL pathogenesis.

Our study also has limitations. Home-collected fecal samples introduce sampling variability. We have addressed this issue in another research paper, which is currently undergoing peer review. We found no differences in either richness or alpha diversity (Shannon index) concerning sampling (from the surface vs. the core of the stool), short-term storage (0–10 days) at room temperature, long-term storage (1, 3, or 6 months) in a freezer (−18 °C or −80 °C), or impact of the buffer medium vs. sterile water. Because of the small size of the cohort, we could only address a limited number of gut microbiome confounders. Information on daily medication was incomplete and relied on admission records, which are known to be incomplete [44]. The sampling container tube collects only 10 mg of fecal matter from the stool's surface, potentially affecting the DNA yield [45]. The rarefaction curves show that some samples' sequencing process was incomplete, which could have affected the results. A shotgun metagenomic approach might have enhanced the precision of the results [46]. Moreover, fecal samples may not fully reflect mucosa-associated microbiota [47], except for rectal mucosal samples [48]. Three patients in the AL group were converted to open surgery, but the influence of this on AL is uncertain [49, 50].

Assessing the power of a microbiome study is difficult due to the high variability between individual microbiomes and the large number of taxa involved. Small changes in abundance may not significantly affect overall diversity, making effect sizes hard to predict. We consider this an exploratory study, and the findings must be validated in a larger cohort. We plan to conduct a follow-up study, hopefully validating our findings in a new and larger cohort and with the inclusion of a regression model.

Future studies should examine to which extent these findings can be replicated in other populations, expand sample sizes, and enhance result precision. Furthermore, the time between sampling and possible surgery could facilitate investigations into microbiome interventions like targeted antibiotics, dietary changes, or fecal transplantation. Moreover, it offers opportunities for in-depth host characterization, including immune system analysis, which could aid surgical decision-making that aims to reduce the risk for AL.

5. Conclusions

We have shown that patients with AL following rectal cancer surgery had decreased alpha diversity and different beta diversity of their baseline microbiome in fecal screening samples compared with matched control patients. Bacteria with the potential to produce collagenase were more common among patients suffering from AL

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CRediT authorship contribution statement

Anders Bech Jørgensen: Writing – review & editing, Writing – original draft, Visualization, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Louise Almer:** Writing – review & editing, Methodology, Formal analysis. **Jose Alfredo Samaniego Castruita:** Writing – review & editing, Visualization, Methodology, Formal analysis. **Martin Schou Pedersen:** Writing – review & editing, Methodology, Formal analysis. **Nikolai Søren Kirkby:** Writing – review & editing, Methodology, Formal analysis. **Esther Agnete Jensen:** Writing – review & editing, Methodology. **Alonzo Alfaro-Núñez:** Writing – review & editing, Methodology. **Lennart Friis-Hansen:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Formal analysis, Conceptualization. **Birgitte Brandstrup:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Data availability

Due to Danish legislation, sharing or storing raw biological information data outside approved Danish institutions is prohibited. You can contact the corresponding author if you have questions about the dataset.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Birgitte Brandstrup reports financial support was provided by The Danish Cancer Society. Birgitte Brandstrup reports financial

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Appendix A. Supplementary data

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