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Mucus Microbiome of Anastomotic Tissue During Surgery Has Predictive Value for Colorectal Anastomotic Leakage

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Objective: The aim of the present study is to investigate the association of gut microbiota, depending on treatment method, with the development of colorectal anastomotic leakage (AL).

Background: AL is a major cause for morbidity and mortality after colorectal surgery, but the mechanism behind this complication still is not fully understood.

Methods: Bacterial DNA was isolated from 123 "donuts" of patients where a stapled colorectal anastomosis was made and was analyzed using 16S MiSeq sequencing. In 63 patients, this anastomosis was covered with a C-seal, a bioresorbable sheath stapled to the anastomosis.

Results: In non-C-seal patients, AL development was associated with low microbial diversity ($P = 0.002$) and correspondingly with a high abundance of the dominant *Bacteroidaceae* and *Lachnospiraceae* families ($P = 0.008$ and 0.010, respectively). In C-seal samples, where AL rates were slightly higher (25% vs 17%), an association with the gut microbiota composition was almost undetectable. Only a few opportunistic pathogenic groups of low abundance were associated with AL in C-seal patients, in particular *Prevotella oralis* ($P = 0.007$).

Conclusions: AL in patients without a C-seal can be linked to the intestinal microbiota, in particular with a low microbial diversity and a higher abundance of especially mucin-degrading members of the *Bacteroidaceae* and *Lachnospiraceae* families. In C-seal patients, however, it seems that any potential protective benefits or harmful consequences of the gut microbiota composition in regard to wound healing are negated, as progression to AL is independent of the initially dominant bacterial composition.

Keywords: anastomotic leakage, colorectal, intraluminal sheet, microbiome, surgery

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Anastomotic leakage (AL) remains the main cause for morbidity and mortality in colorectal resection surgery, leading to prolonged hospital stays and significant costs.[1–3] Although surgical factors as perfusion of and tension on the anastomosis and patient-related factors as comorbidity and medication are known factors, in many cases no explanation can be given for the failure of anastomotic healing.

It is well recognized that the gut microbiota plays an important role in human health, and an expanding list of diseases has been associated with the microbial composition and/or their products.[4] Intestinal diseases, in particular, have been associated with the intestinal microbiota.[4–6] Products produced by bacteria, such as short-chain fatty acids (SCFAs), are important for colonic cells. Butyrate, for example, is the primary energy source for colonic cells.[7] In addition to its importance in the defense against opportunistic pathogens, the glycoproteins in the mucus layer (mucin) also serve as a source of nutrients for commensals/symbionts such as the anti-inflammatory butyrate-producing bacterium *Faecalibacterium prausnitzii*.[8] However, when the supply of butyrate to the colon is diminished or stopped, the colonic mucosa may enter a state of energy deprivation, leading to colitis and diarrhea.[9,10] Furthermore, the colonic microbiota is also important in regard to wound healing.[11] In addition, selective decontamination of the digestive tract reduces infections and seems to have a beneficial effect on AL in colorectal surgery.[12]

In a previous pilot study,[13] we investigated the possible role of colonic microbiota in AL using samples from 8 patients who developed AL matched with 8 patients without AL who were included in the C-seal trial[14] but who were not treated with a C-seal. We found that an overabundance of bacteria from *Lachnospiraceae* family and low microbial diversity were linked to AL development.

The aim of the present exploratory study is to investigate the role of the gut microbiota using 16S rRNA analysis, in the development of AL in greater detail using a larger group of patients, and to analyze whether the use of a C-seal during treatment, an intraluminal sheet originally designed for the protection of the anastomosis,[14] influences the role between the gut microbiota composition and AL development.

METHODS

The methods used for this study are the same as described in the previously performed pilot study.[13]

Patients

Twenty-nine patients who developed AL were matched on sex, age, and preoperative chemotherapy and radiotherapy with 94 patients without AL. AL was defined as AL leading to a reintervention. All patients participated in the C-seal trial, a trial to evaluate the effect of the C-seal in the prevention of clinical AL in the stapled colorectal anastomosis. This multicenter trial was designed to evaluate the efficacy of the C-seal; the primary endpoint was AL requiring reintervention. This trial was open for inclusion from December 2011 until January 2014.

The study was approved by the Medical Ethics Committee of the University Medical Centre Groningen and all participating

centers. The trial was registered in The Netherlands National Trial Register under the number NTR3080. In total 539 patients were included; all patients provided written informed consent; and additional consent was asked to retrieve and store the circular stapler donuts. All data were collected anonymously, encoded, and saved in a database.

Sample Collection

Bacterial DNA of the available proximal donuts was isolated and subsequently analyzed using MiSeq sequencing of the amplified 16S rRNA genes. The reason for studying 16S rRNA genes using MiSeq sequencing is because all bacteria have 16S rRNA genes, and the small differences in their 16S rRNA genes allow us to identify all the microbial groups present within a sample. Sequencing allows us to quantitatively analyze the relative abundance of all species, including species which we are yet unable to culture in the laboratory. The often complex bacterial composition of a sample, including the analysis of more rare low-abundant bacteria, can hence be measured in a much more cost-effective and accurate fashion than was possible with previous microbiome classification methods such as fluorescent in situ hybridization microscopy counting techniques.

DNA Extraction and MiSeq Preparation

Total DNA was extracted, as described by de Goffau et al,[15] from 0.25 g of a "donut." Care was taken not to include any macroscopic traces of stool. The additional purification steps using columns were not needed after DNA precipitation. The V3 to V4 region of the 16S rRNA gene was amplified from the DNA by PCR using modified 341F and 806R primers with a 6-nucleotide barcode on the 806R primer. The sequence of the 341F primer and the 806R primer was aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctNNNNCCTACGGGAGGCAGCAG and caagcagaagacggcatacgagat**CGTGAT**gtgactggagttcagacgtgtgctcttccgatctGGACTACHVGGGTWTCTAAT, respectively, where lowercase letters denote adapter sequences necessary for binding to the flow cell, underlined lowercase letters are binding sites for the Illumina sequencing primers, bold uppercase letters highlight the index sequences as reported by Bartram et al,[16] and regular uppercase letters are the V3 to V4 region primers (341F for the forward primers and 806R for the reverse primers). The inclusion of 4 maximally degenerated bases (NNNN) maximizes diversity during the first 4 bases of the run. A detailed description of the PCR, DNA cleanup, and MiSeq library preparation, as described by Heida et al,[17] are found in Supplementary Data file 1, http://links.lww.com/SLA/B359.

MiSeq Sequencing Pipeline and Statistical Analysis

Software that was used to analyze the data received from Illumina paired-end sequencing included PANDAseq,[18] QIIME, and ARB.[19] Reads with a quality score <0.9 were discarded by PAN-DAseq. Statistical analyses were performed on the family, genus, and species level. QIIME identified sequences down to the family and genus level and was used to perform weighted alpha-diversity analyses, whereas ARB was used to identify sequences down to the species level. Principal component analysis (PCA) was performed to describe the variation in all the bacterial groups into a very limited amount of new relevant dimensions of variability to address the issue of multiple testing. The hierarchical clustering analysis was performed with the Hierarchical Clustering Explorer version 3.0 (http://www.cs.umd.edu/hcil/ multi-cluster/). Percentages (%) given of a microbial group in a group of patients indicate the average percentage of reads assigned to that group. The Simpson index was used as a measure of microbial diversity. Nonparametric tests were used, as microbial abundances are rarely normally distributed and are preferred as they are more conservative. Mann–Whitney

U or Spearman's ρ tests were used as indicated. The use $\pm$ indicates that a standard deviation is given. All tests were two-tailed, and a $P <$ 0.05 was considered to indicate statistical significance. All statistical analyses were performed using IBM SPSS Statistics 20.0.

RESULTS

Of 123 samples in total, 122 were included in the PCA, as 1 sample had an insufficient amount of bacterial DNA (Table 1). However, 3 C-seal AL patients and 1 non-C-seal AL patient were excluded from subsequent statistical analyses, as clear nonmicro-biota-related reasons were found why these patients developed AL; 3 had necrosis of the proximal bowel loop and 1 had a technical failure of the C-seal.

Surprisingly almost no difference was found between AL and non-AL patients when the 118 samples were analyzed together; only the *Blautia* genus was more abundant among AL patients ($P =$ 0.040). However, when we looked at the subgroup of non-C-seal patients, the microbiota of AL versus non-AL was different.

Sixty samples were from patients who were randomized to the C-seal group and 58 were from the group without a C-seal. Of the 60 C-seal patients, 15 developed AL, whereas this number was 10 of the 58 in the non-C-seal patients. In a comparison of the C-seal samples with all the non-C-seal samples, no statistically relevant differences were found in bacterial compositions either on the genus or family level. However, AL patients without a C-seal had a much lower microbial diversity ($P = 0.006$), more *Bacteroides* ($P = 0.006$), more *Lachnospiraceae* ($P = 0.05$), and less *Prevotella* ($P = 0.05$) and *Streptococci* ($P = 0.03$) than C-seal patients who developed AL. All other patient characteristics were, in comparison with the presence or absence of a C-seal, irrelevant. Striking differences were subsequently revealed between C-seal samples and non-C-seal samples in regard to AL and non-AL samples.

TABLE 1. Patient Characteristics

	AL (n = 29)	No AL (n = 94)	P
C-seal/no C-seal	18/11	45/49	0.207
Sex male/female	22/7	61/23	0.365
Age, y (SD)	63.4 (10.4)	63.4 (10.4)	0.510
Body mass index (SD), kg/m²	26.0 (3.9)	26.9 (4.0)	0.075
Charlson comorbidity index			0.415
0	16	56	0.830
1	5	23	0.462
2+	8	15	0.179
Indication for surgery			
Colorectal cancer	26	91	
Diverticular disease	2	2	
Other	1	1	
Preoperative treatment			
Radiotherapy			
No radiotherapy	13	39	0.831
Short course	11	28	0.494
Long course	5	27	0.240
Chemotherapy	5	30	0.160
Corticosteroid use	1	3	0.999
Deviating ostomy present	1	9	0.449
ASA-score			
1	7	19	0.795
2	17	64	0.376
3	5	11	0.528

All patients received mechanical oral bowel preparation and antibiotics prophylaxis.

FIGURE 1. Principal component analysis (PCA) plot of all 122 samples, divided over all 4 groups and relevant associations. PC1, represented by the *x*-axis, is associated with AL in non-C-seal patients and describes 58% of the variation in the data. PC3, represented by the *y*-axis, is positively associated with microbial diversity and negatively associated with AL and describes 6% of the variation in the data. Vectors in the upper right corner represent the correlation coefficients of the respective variables with PC1 and PC3. Colored vectors correspond to the AL and C-seal status as indicated in the legend. In non-C-seal patients, the AL cases nearly without exception found in the lower bottom corner, as indicated with a dashed red circle, which is indicative of a microbiota dominated by *Lachnospiraceae* and *Bacteroidaceae* of low diversity. Non-C-seal controls are more commonly found to have higher scores on PC3, which is associated with a higher microbial diversity, containing more *Ruminococcaceae*, more *Prevotella copri*, and/or more *Streptococcaceae*.
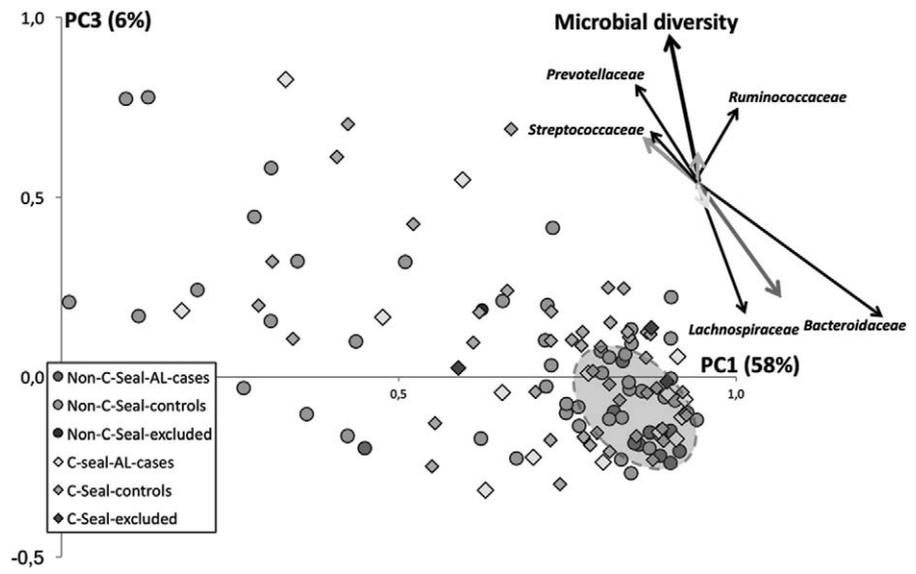
Principal Component and Hierarchical Clustering Analysis

A PCA plot showed all 122 samples, divided into 4 main groups based upon C-seal status and AL occurrence, combined with a correlation analysis of the main microbial groups and microbial diversity (Fig. 1). This highlights some of the main differences between C-seal patients and non-C-seal patients with respect to clinical outcome. The most striking aspect of Figure 1 is how nearly all non-C-seal AL samples cluster together in the lower right corner as indicated with a red dashed circle, whereas the other 3 groups have a seemingly identical distribution. Correlation analyses confirm that the clustering/scattering of C-seal AL samples and C-seal non-AL samples is almost identical, indicating that in the C-seal patients the dominant microbial composition of the samples is unlikely to be related to the development of AL. The distribution of non-C-seal non-AL samples at first glance seems to be similar to the distribution of C-seal patients, yet correlation analyses show that these samples tend to be more located to the upper left. Non-C-seal AL samples score higher on PC1 ($P = 0.012$) and lower on PC3 ($P = 0.0006$) than non-C-seal non-AL samples. The localization of samples in the lower right corner is associated with a bacterial composition that is strongly dominated by *Lachnospiraceae* and/or *Bacteroidaceae* and that is consequently low in microbial diversity.

A hierarchical clustering analysis on the C-seal samples in combination with a microbial profile at the family level yet again showed a homogenous distribution in both AL and non-AL samples (data not shown). However, when this technique is applied to the non-C-seal samples, a cluster of samples is found within the hierarchical clustering in which AL samples are overrepresented (7/14 vs 3/44, $P = 0.0002$) and in which *Lachnospiraceae* are dominant (40%) followed by *Bacteroidaceae* (28%) (Supplemental Figure 1, http://links.lww.com/SLA/B359). Furthermore, a microbial profile of non-C-seal samples with a focus on both the microbial diversity, which is lower in the AL samples ($P = 0.002$), and the combined prevalence of *Lachnospiraceae* and *Bacteroidaceae* underscores the strong association of these family groups (Fig. 2) with the occurrence of AL.

Taxonomic Analysis

Individual analyses of taxonomic groups on the phylum, family, genus, and species level in the case of C-seal samples again highlight the apparent irrelevance of the microbiota composition in regard to the occurrence of AL. Only the presence or absence of a few low-abundant opportunistic pathogenic groups was found to be almost exclusively (weakly) associated with AL in C-seal samples. These included the *Tenericutes* phylum (11/15 vs 19/45, $P = 0.037$, chi-square test), the *Leptotrichia* family (8/15 vs 9/45, $P = 0.013$), and *Prevotella oralis* (7/15 vs 6/45, $P = 0.007$). The abundance of *Bacteroides uniformis* and *Bacteroides ovatus* were found to be negatively associated with the occurrence of AL in C-seal patients (0.8% vs 2.5%, $P = 0.001$ and 0.9% vs 0.4%, $P = 0.01$). Of the different *Bacteroides* species, *B. uniformis* was also the most negatively correlated with *P. oralis* ($P = 0.008$).

Differences between AL and non-AL in the non-C-seal group were much more abundant. The *Lachnospiraceae* family is associated with AL (40% vs 27%, $P = 0.010$), and consists of multiple important genera of which the *Blautia* genus (8% vs 4%, $P = 0.003$), in particular *Blautia obeum*, is the most strongly associated with AL (7% vs 3%, $P = 0.005$). The *Bacteroidaceae* family is furthermore associated with AL (28% vs 17%, $P = 0.008$). On the contrary, *Prevotella copri* and the *Streptococcus* genus are both negatively associated with AL development in non-C-seal patients (Tables 2 and 3). *P. copri* was completely absent in 8/10 of the AL cases, whereas it was absent in only 11/48 of the non-AL cases ($P = 0.0005$, chi-square test).

Predictive Analyses

As Figure 1 and Supplemental Figure 1, http://links.lww.com/SLA/B359 show, AL cases of non-C-seal patients seem to be almost without exception dominated by *Lachnospiraceae* and *Bacteroidaceae* with correspondingly low microbial diversity scores. As a measure for future predictive analyses, we defined a set of criteria to describe a microbial composition that predisposes patients to developing AL after surgery. These criteria were chosen as such
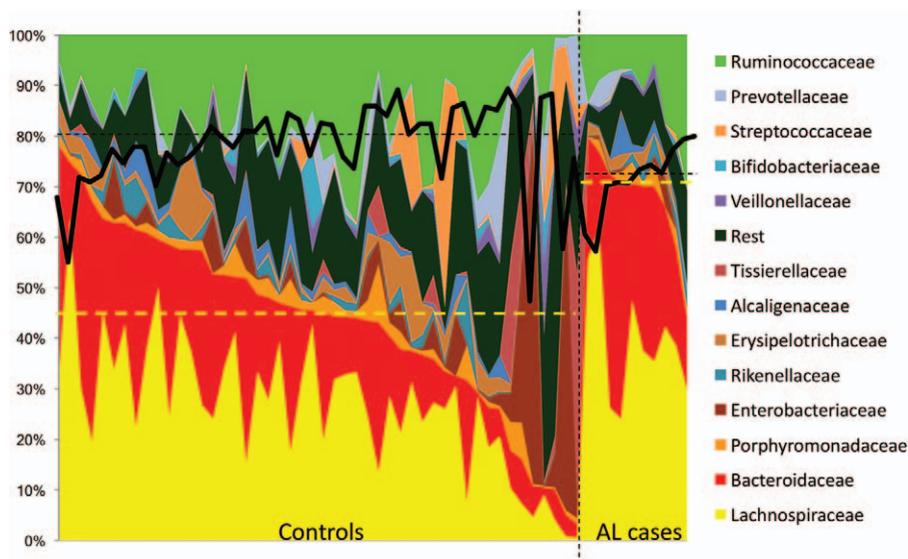
FIGURE 2. Microbial composition profile of non-C-seal patients. AL cases are depicted to the right of the dashed vertical line and controls are on its left. The dashed horizontal black lines represent the median simpson index value (%/100) of controls (left) and AL cases (right) and show that the diversity is higher in controls ($P = 0.002$). The orange lines represent the median values of the sums (%) of the 2 most dominant bacterial families, *Bacteroidaceae* and *Lachnospiraceae*, and show that this sum is on average lower in controls than in AL cases [$P = 0.008$ and 0.010 separately, respectively, for both families and $P = 0.0002$ when both families are combined (orange lines)].

that an approximately equal number of patients from the C-seal and the non-C-seal patient cohort would meet these criteria. Samples were prone to developing AL if the total sum of *Lachnospiraceae* and *Bacteroidaceae* in them was higher than 60% and when the Simpson diversity score on the family level was <0.75. Eight out of 14 samples from the non-C-seal group who met these criteria developed AL. For the C-seal group, this was only 3 out of 13. The odds ratio for developing AL when meeting the criteria as defined above was 0.9 for the C-seal group ($P = 0.9$), but for the non-C-seal group this was 28 ($P = 0.00001$).

DISCUSSION

This study shows a relation between the composition of the intestinal microbiota and the subsequent development of AL after stapled colorectal anastomoses, but only in patients who underwent surgery without the additional C-seal that covered the anastomoses. In a previous pilot study on AL, we analyzed 16 non-C-seal patients of whom 8 developed AL.[13] The present study included an additional

TABLE 2. Main Associations With AL in Non-C-seal Samples (MW-U Test)

Reduced Risk	Average, %	P
Prevotella copri	1.0	0.007
Streptococcus genus	2.6	0.012
Streptococcus salivarius	0.5	0.018
Eubacterium biforme	1.5	0.010
Increased risk		
Lachnospiraceae	29	0.010
Blautia genus	4.9	0.004
Blautia obeum	3.8	0.005
Blautia glucerasei	0.7	0.014
Ruminococcus torques	1.4	0.029
Coprococcus	5.9	0.098
Roseburia	3.7	0.094
Bacteroidaceae	19	0.008
Bacteroides	19	0.028
Bacteroides fragilis	3.2	0.013

63 C-seal and 44 non-C-seal patients, with 2 additional leakages in the non-C-seal group.

Non-C-seal

In this larger group of non-C-seal samples, the correlations with AL confirm most of the results we found in the pilot study, as a high abundance of *Lachnospiraceae* and *Bacteroidaceae* and a lower microbial diversity are still strongly associated with AL. A bacterial composition that consists of 60% or more of these 2 families seems predictive for AL.

The trophic network of species in intestinal microbiota with a low diversity may be more easily disturbed than in microbiota with a high diversity.[20] This disturbance could be provoked by preoperative or surgical processes, such as intravenous antibiotics, mechanical bowel preparation, the creation of deviating ostomies, opioids, or even the impact of the surgery itself.[20–23] A disturbed microbial composition may affect the metabolic balance; a reduction of butyrate production might, for instance, initiate energy deprivation, causing impaired functioning of the colonic cells and their ability to heal. It has been found in rats that an intraluminal infusion of SCFAs resulted in significantly stronger colonic anastomoses.[24] Rectal irrigation with SCFAs in humans with ulcerative colitis or diversion colitis has also shown promise.[9,25] Furthermore, a disturbed microbiota of low diversity may lack colonization resistance to pathogenic bacteria that could play a role in the development of AL, for example, *Enterococcus faecalis*.[26–28] It would be very interesting to compare the microbiota at the time of surgery with the microbiota at the time of AL.

In this study, the focus in the non-C-seal samples seems to be on the importance of microbial diversity and possibly on the role of mucin degradation, possibly with an important mediating role of the *Ruminococcaceae* family, which contains a high number of important butyrate-producing species such as *F. prausnitzii*. Of the 3 most dominant microbial families in the gut, *Bacteroidaceae* (19%), *Lachnospiraceae* (29%), and *Ruminococcaceae* (16%), the first 2 are strongly negatively correlated with microbial diversity. Both *Bacteroides* and *Blautia* (from the *Lachnospiraceae* family) are known mucin-degraders that mainly either produce acetate and propionate or propionate and propanol, but neither of them produces butyrate.[29,30] Despite their high prevalence, *Ruminococcaceae* are

TABLE 3. Defined Characteristics Included Samples

	C-seal (n = 63)		Non-C-seal (n = 59)	
	Control (n = 47)	AL (n = 15)	Control (n = 49)	AL (n = 10)
Sex (male/female)	24/23	10/5	37/12	8/2
Age, y (SD)	63.3 (10.3)	66.8 (12.0)	53.3 (10.7)	62.2 (8.9)
Body mass index (SD), kg/m^2	27.8 (5.3)	27.4 (3.0)	26.5 (3.7)	29.5 (5.7)
Average amount of comorbidities	1.8	2.3	1.6	2.6
Indication for surgery				
Colorectal cancer	45	15	45	10
Diverticular disease	1	0	3	0
Other	1	0	1	0
Preoperative treatment				
Radiotherapy				
No radiotherapy	20	5	21	5
Short course	12	7	16	3
Long course	15	3	12	2
Chemotherapy	15	3	15	2
Corticosteroid use	0	0	3	1
Deviating ostomy present	4	0	7	1
ASA-score				
1	8	2	11	2
2	32	11	31	5
3	4	2	7	2

strongly positively associated with microbial diversity, especially the metabolically highly important keystone species *F. prausnitzii* (7%) and *Ruminococcus bromii* (3%).[31]

C-seal

In the C-seal trial, we found a trend to more AL in C-seal patients than in non-C-seal patients.[32] However, the overall microbial composition in C-seal patients does not seem to play a role in the occurrence of AL at first. Our observations suggest that the C-seal influences the microbial composition after introduction. This may be due to the barrier it creates between the mucosa and the (fresh) luminal content, interrupting the supply of new resources. The subsequent reduced rate of metabolism (SCFA production), possibly reduces the rate of mucin synthesis by the human host, negatively affects wound healing.[33] The C-seal may create a new ecosystem that benefits the growth of potential opportunistic pathogens as seen in our analysis, like *P. oralis, Fusobacteriacea, Leptotrichiaceae,* bacteria from the phylum *Tenericutes,* and *Enterococci* as seen by others,[20] represent (if at all present) a very small minority, but could perhaps prosper and subsequently increase inflammation in this new situation.

Another ecological factor might be that shielding off the mucosa, and the subsequent lack of metabolism, makes the environment more aerobic. As the metabolism diminishes, oxygen diffusing from the blood into the lumen is utilized less rapidly,[34] making life hard for commensal oxygen sensitive species while facilitating growth for opportunistic facultative pathogens, such as *Enteroccocus* species, which are shown to excrete gelatinase GeIE causing degradation of the anastomotic tissue.[20]

Strengths and Limitations

Most of the information that is available on the composition of the gut microbiota is derived from fecal samples. This reflects the composition present in the lumen of the distal colon and rectum, but is different from the composition in the mucosa.[35] The bacterial DNA from the mucus layer was isolated in this study, giving a much better insight on the microbial composition around the anastomosis than a fecal sample could provide. Although all patients had oral mechanical bowel preparation and the intestine was checked on residual luminal content, we cannot guarantee that in some of the samples small traces of luminal content might have been present.

The method used to identify the bacterial DNA has its limitations because 16S rRNA sequencing can only detect relative abundance and not the absolute density of bacterial DNA present in the mucus. In addition, 16S analysis is only capable of identifying particular species based upon their 16S rRNA gene; genetic variation within species cannot be measured. Some species, like *Escherichia coli,* have a huge genetic diversity, distinguishing pathogenic *E. coli* from nonpathogenic *E. coli* is impossible with 16S analysis. Furthermore, to confirm our hypotheses, our data should have included the mucosal microbiome of the patients after the surgery, and, ideally, after the development of AL. As this is very difficult, alternatively, fecal samples could be collected both before and after surgery, in addition to the "donut" sample taken during surgery.

Regarding the prevention of AL, we would recommend future research to be focused on altering the gut microbiota by diet before surgery into another stable yet healthy low risk composition, favoring *Ruminococcaceae, P. copri,* and a high microbial diversity.

CONCLUSIONS

The microbial composition in patients that underwent standard colorectal surgery has a predictive value in regard to whether they develop AL or not. Patients seem to have a higher risk of developing AL when their microbial diversity is low, which in turn is often associated with an overabundance of members from the mucin-degrading families *Lachnospiraceae* and *Bacteroidaceae.* The introduction of a C-seal, however, completely negates the protective or harmful consequences of the dominant gut microbiota before surgery in regard to wound healing. Further studies should be conducted to elicit the possible mechanisms between the microbial composition and the development of AL.

ACKNOWLEDGMENTS

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