Elucidating the mechanisms of anastomotic leakage

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Elucidating the Mechanisms of Anastomotic Leakage

A New Point of View

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A New Point of View

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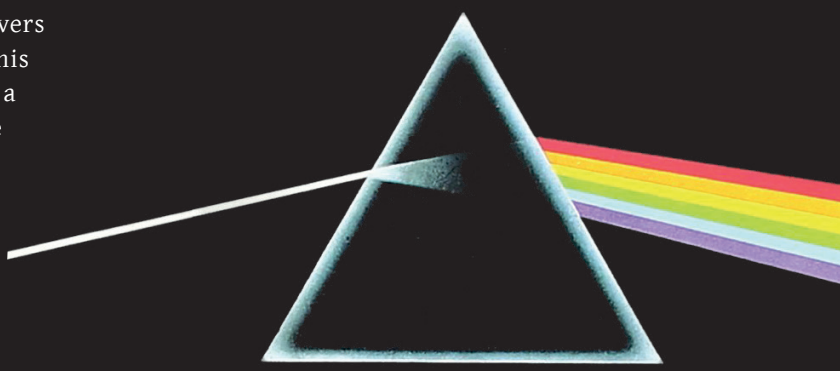
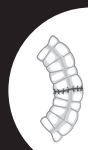
OUTLINE

THE DARK SIDE OF THE MOON

A legendary album by the British band Pink Floyd, with one of the most recognized album covers of all time. The songs in this album can together be seen as a cautionary tale, which is a tale told to warn its listener of a danger. The dark side of the moon is also an expression used to describe something unknown or mysterious. It's also simply an epic album.





CHAPTER 1

GENERAL INTRODUCTION AND OUTLINE OF THIS THESIS

GENERAL INTRODUCTION

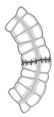
Annually, more than a million new colorectal tumours are diagnosed worldwide, making it the fourth most common cancer. (1) Approximately 30% of these tumours are located in the rectum. (2,3) The overall incidence of colorectal tumours has steadily increased over the last decades. The highest incidence rates are in Australia and New Zealand, Europe, and North America and are up to a 10-fold higher than in non-western countries. (4) This difference is often assigned to the western lifestyle: not enough physical activity, too much (processed) food full of fat and sugars and with a low amount fibre. (5,6) The high incidence rate has led to successful (secondary) prevention initiatives such as colonoscopy and stool analysis. (7,8)

Surgery is the cornerstone of colorectal cancer treatment; in advanced cases this is combined with neoadjuvant chemotherapy and/or radiation therapy. Tumour resection with the construction of a colorectal anastomosis is usually the preferred surgical approach, as it averts a permanent ostomy. (9) The creation of anastomoses in patients undergoing colorectal surgery has been an art for centuries and various techniques have been described. With every technical variation the construction of the anastomosis was optimised in order to prevent the feared complication of anastomotic leakage.

Primary anastomosis

At first, the treatment of wounds of the intestines was a *noli (me) tangere* (do not touch) for even the most famous surgeons, under the belief that nature's resources would prove more successful in saving the life of the patient than a surgical intervention would. (10) Fortunately, along with the knowledge about antisepsis and asepsis this belief has changed over time. Although a bowel resection was rarely performed in ancient surgery, restoring continuity of the intestine was already practiced with a wide variety of techniques long before the 19th century. In the more recent history, anastomoses were created. These were performed with various techniques, such as inverted/everted/circular and single or double layered suturing, and diverse degrees of complications. These surgical techniques have greatly improved over the past three centuries, and postoperative complication rate have fallen accordingly. However, the occurrence of post-operative anastomotic leakage has been a persistent problem.

To date anastomotic leakage is one of the most common surgical complications after colorectal resection. Leaking of the anastomosis causes intraluminal (faecal) content, to excavate to the normally sterile abdominal cavity. It leads to high rates of morbidity, re-interventions, prolonged hospital stay and increased mortality. (11-13) In addition, it may worsen oncological outcome. (14) There seems to be an increased rate of local recurrence and decreased disease-free survival in patients that develop



an anastomotic leak. (15,16) The incidence of leakage varies largely, partially because the definition of anastomotic leakage is still under debate. (17,18)

Even though the cause for anastomotic leakage is often unknown, the percentage of anastomotic leakage after colorectal surgery is seen as a parameter for quality of care or as an indicator for the surgeon's skill. In some cases, anastomotic leakage is caused by technical imperfect anastomosis, tension on the anastomosis and decreased vascularization of the bowel. Many risk factors have been established, including patient factors as comorbidity, American Society of Anaesthesiologist classification and use of medication. (19-21) Other, more biological factors like the level of inflammation markers procalcitonin or C-reactive protein and the calcium score, (22-26) peritoneal fluid biomarkers including cytokines (27) and non-steroidal anti-inflammatory drugs (NSAIDs) are known to play a role in the development of anastomotic leakage since the COX-2 enzyme is essential for the healing. (28,29) Although many influencing factors involving the development of anastomotic healing have been established, these factors don't explain the mechanism of failure of the anastomotic healing.

Intestinal wound healing

The exact mechanism of effective wound healing in the gastrointestinal tract isn't known yet. However, it can be assumed that intestinal wound healing has the same stages as wound healing processes elsewhere in the body. Wound healing can be divided in roughly three phases: inflammation, proliferation and remodelling. (30) Inflammation is the initial response that facilitates haemostasis and the immunological defence. The second phase, proliferation, is triggered by growth factors secreted by macrophages. This stage is characterized by angiogenesis, epithelial cell migration and influx of fibroblasts. Remodelling of the wound healing process mainly exists of collagen restructuring. (30) There are two main types of wound healing, primary and secondary. Primary wound healing (or wound healing with primary intention) occurs when the wound edges are closely approximated. Secondary wound healing is healing with the edges of the wound not well-approximated. Anastomotic healing is secondary wound healing, as the wound edges are inverted into the lumen. Therefore, anastomotic healing starts with granulation, the transformation of clotting matrix and thus collagen formation, which is needed for gapping the wound defect. This causes the balance of collagen production and degradation to be an essential part of the healing of the anastomosis. (31-33) The fusion of the submucosal collagen matrix, which provides strength to the bowel, is essential for good intestinal wound healing. (34)

Multiple strategies to reduce the incidence of anastomotic leakage have been suggested and tested. A delayed anastomosis, by creating an end-colostomy and

postponing the creation of an anastomosis doesn't reduce the leak rate. (35,36) Other techniques, including medical devices as the "Murphy's button" (1892), (37) transanal stents (38) or intraluminal devices (e.g. Coloshield) have been used but eventually (mostly) discontinued in their use or do not alter the clinical anastomotic leak rate. (39,40) The effect of a diverting ostomy or an omentoplasty is still under debate. (41,42)

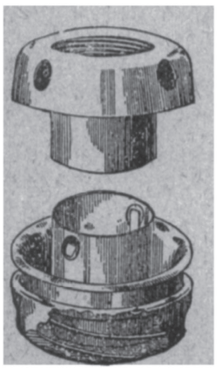


Fig. 50. Murphy's button.

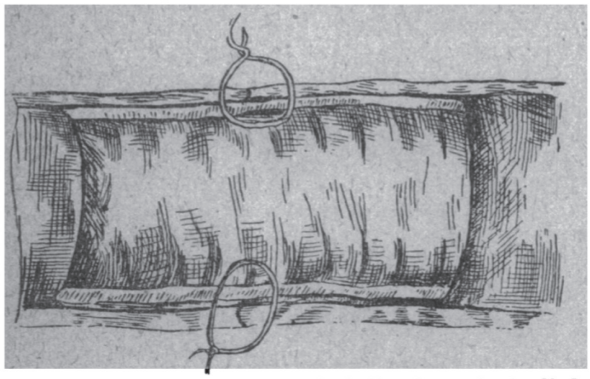


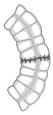
Fig. 3. Suture of Du Verger. Sutures including the tracheal cylinder.

Figure 1 (fig 50.) An illustration of the Murphy's button and **(fig 3.)** an impression of the intraluminal application of a tracheal cylinder to restore continuity (derived from *Senn, JAMA 1893*(10))

Interestingly, a dried trachea (Saliceto, 1520) or even a cylinder of cardboard besmeared with sweet oil, essence of turpentine or oil of St. Johnsworth (Du Verger, 17th century) was used to help restoring continuity and make sure intraluminal content wouldn't excavate (see figure 1, derived from Senn, 1893). (10) Our modern-day effort similar to trachea or cardboard was the C-seal, a biodegradable intraluminal device designed for the protection of the anastomosis. It was stapled along with the anastomosis and would fold over the anastomotic wound (see figure 2). First (43) and second (44) phase trials were very promising, which made way for a multicentre randomized clinical trial. (45) During this trial, we observed stercoral perforation (due to the necrotizing pressure of faecal impaction) in several cases, (46) after which the protocol had to be changed. Unfortunately, creating an anastomosis with the application of a C-seal, did not show a reduction in leakage-rate compared to a regular stapled anastomosis. (40)

Secondary wound healing and microbiota

The healing of the anastomosis is based on secondary wound healing in an incredibly "dirty" environment. It has been a longstanding practice to keep bacteria away from any other wound, but we only recently started to look critically at the bacterial



influences on the intestinal wound healing. The idea of involvement of bacteria dates from the 50's of the previous century, where antibiotic application at the site of an anastomosis in an animal-model showed normal wound healing instead of leakage and peritonitis. (47) Since then the leakage rate has shown to decrease, but not eliminated, by the use of oral non-absorbable antibiotics. (48) The recent development of advanced techniques has made studies on microorganisms and their capabilities easier and cheaper. Using these techniques, it was established that bacterial species with collagen degrading aspects can cause anastomotic leakage in rodent models. (32,49)

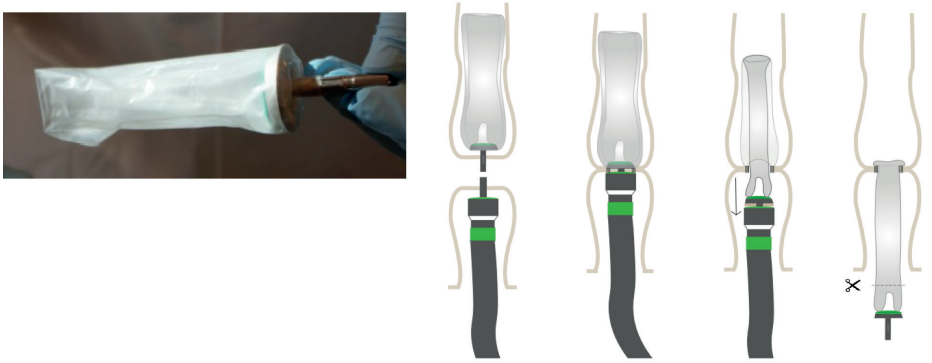


Figure 2 (left) the C-seal (45) and **(right)** the application of the C-seal with the creation of a stapled colorectal anastomosis. (46)

Recurrence of tumour

Of all colorectal cancer patients, 16% will develop a local malignant tumour after an attempted curative resection. (50-52) It is known that cancer cells are continuously shed into the lumen, before, during and after colorectal cancer resection. (53,54) However, most of these recurrent tumours appear outside of the intestinal lumen. They are adjacent to anastomotic tissue and are usually not detected by surveillance endoscopy. (55) Although the mechanism behind this hasn't been established yet, interestingly the recurrence of colon cancer is higher in patients that consume a western diet. (56) The western diet, a major cause of obesity, in turn has been associated with immense changes in the composition of the intestinal microbial composition. (57-59) As many other diseases have been associated with the intestinal microbial composition, this opens the speculation for an influence of the intestinal microbiome on the development of cancer (and possibly cancer recurrence). (60-65)

OUTLINE OF THIS THESIS

This thesis aims to elucidate the mechanisms behind anastomotic leakage. The scientific results presented in this thesis are in part an effluent of the C-seal trial and the samples obtained during this trial.

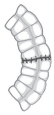
Although the C-seal trial wasn't able to proof the efficacy of the C-seal, a large number of samples was obtained during this trial. The samples that were retrieved were the doughnuts from the circular stapler. This 'doughnut' is the small ring of colon and/or rectum that is cut by the circular stapler when the anastomosis is made. It is a good representation of the tissue at the site of the anastomosis and is taken during surgery. These samples were used in *Chapter 2, Chapter 3* and *Chapter 5.*

With new techniques, such as next generation sequencing, a whole new area of research had become within reach. These techniques caused a rise in amongst others 16S rRNA gene sequencing and thus in microbial research in health and disease. However, the role of the intestinal microbiota in surgical complications such as anastomotic leakage wasn't addressed yet. Therefore, an effort was made to see whether the intestinal microbiome might play a role in the development of anastomotic leakage. In *Chapter 2* a pilot study on the feasibility of microbiome 16S rRNA gene analysis on peroperative doughnut samples is delineated. This pilot study showed that the doughnut samples could be used for further research, Therefore, *Chapter 3A* describes the subsequent 16S rRNA gene study on a larger number of samples. This study raised questions, partly because of the novelty of this methodology in the surgical field. Therefore, an additional clarification of the data is described in *Chapter 3B.* In addition to that, *Chapter 4* is a review about culture-independent microbial research and should serve as a guide for the surgical researcher interested in microbial research.

Because there's an assumed interaction between host and bacteria, it would be interesting to see whether there are molecular differences between patients with and without development of AL. In *Chapter 5 a* gene transcriptome study is presented, identifying patients' gene expression profiles and their pathway co-functionality of the previously used samples.

Research by dr. Alverdy at the University of Chicago has shown that certain bacteria possess certain virulence factors that have the ability to cause anastomotic leakage. These bacteria are *Enterococcus faecalis, Pseudomonas aeruginosa* and *Serratia marcescens.* (32,49) In *Chapter 6* an additional species is added to that list. In addition, this chapter describes a workflow for the detection of this "leak phenotype".

In *Chapter 7* other species are described to have the leak phenotype and also show to have influence on tumour recurrence in a model for tumour recurrence at the site of the anastomosis. In *Chapter 8* all previous chapters are summarized and discussed.



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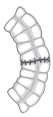

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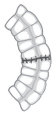
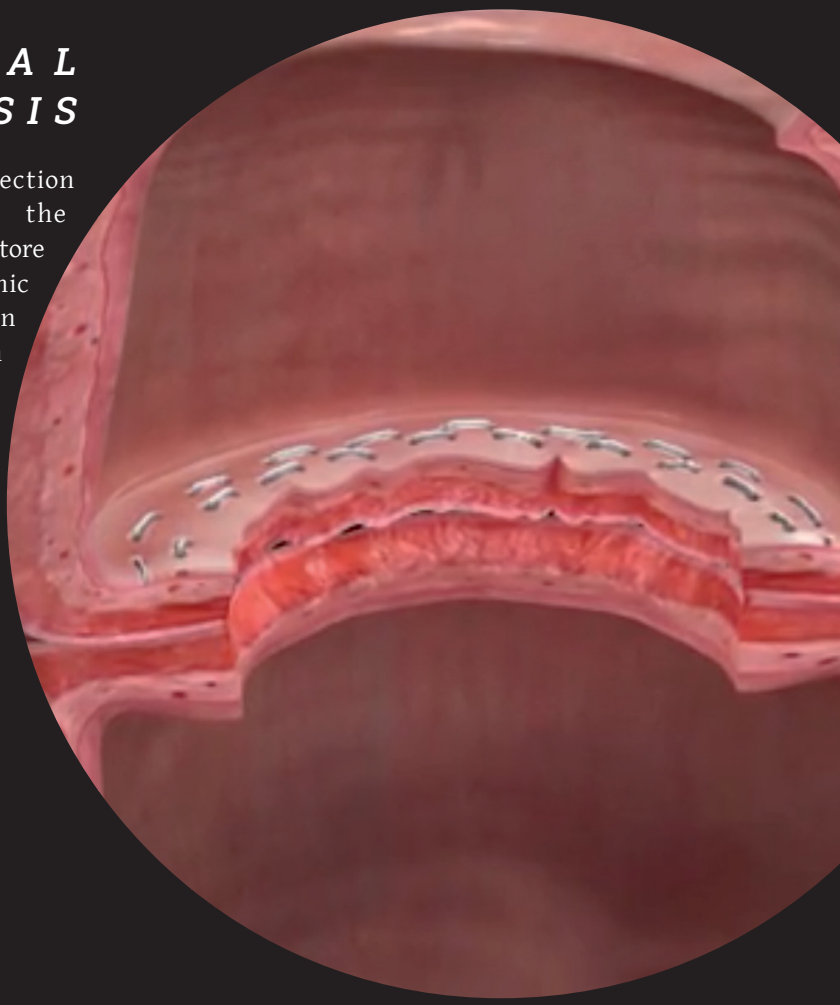
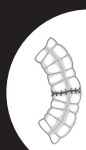


COLORECTAL ANASTOMOSIS

A surgically created connection between the colon and the remainder of the rectum to restore the continuity of the colonic tract (large bowel). This can be done with a handsewn or a (circular) stapled technique. Anastomoses can theoretically be made in the entire gastrointestinal tract. Unfortunately, these anastomoses often leak...





CHAPTER 2

INTESTINAL MICROBIOTA AND ANASTOMOTIC LEAKAGE OF STAPLED COLORECTAL ANASTOMOSES: A PILOT STUDY

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ABSTRACT

Background

Anastomotic leakage (AL) after colorectal surgery is a severe complication, resulting in morbidity, reinterventions, prolonged hospital stay and, in some cases, death. Some technical and patient-related aetiological factors of AL are well established. In many cases, however, none of these factors seem to explain the occurrence of AL. Recent studies suggest that the intestinal microbiome plays a role in wound healing, diabetes and Crohn's disease. The aim of this study was to compare the intestinal microbiota of patients who developed AL with matched patients with healed colorectal anastomoses.

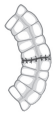
Methods

We investigated the microbiome in the doughnuts collected from 16 patients participating in the C-seal trial. We selected eight patients who developed AL requiring reintervention and eight matched controls with- out AL. We analysed the bacterial 16S rDNA of both groups with MiSeq sequencing.

Results

The abundance of *Lachnospiraceae* is statistically higher (P = 0.001) in patient group who did develop AL, while microbial diversity levels were higher in the group who did not develop AL (P = 0.037). Body mass index (BMI) was also positively associated with the abundance of the *Lachnospiraceae* family (P = 0.022). Conclusion A correlation between the bacterial family *Lachnospiraceae*, low microbial diversity and anastomotic leakage, possibly in association with the BMI, was found. The relative abundance of the *Lachnospiraceae* family is possibly explained by the higher abundance of mucin-degrading *Ruminococci* within that family in AL cases (P = 0.011) as is similarly the case in IBD.

INTRODUCTION



Anastomotic leakage (AL) after colorectal surgery is a severe complication, resulting in morbidity, reinterventions, prolonged hospital stay and, in some cases, death. AL can be defined as a defect of the integrity of the intestinal wall at the anastomotic site leading to a communication of the intra- and extraluminal compartments. It may present as a subclinical abscess that drains spontaneously and needs no further treatment to a completely dehiscent anastomosis leading to a faecal peritonitis and sepsis. In many cases a temporary or definitive ostomy is made. A nationwide Dutch audit revealed an AL rate (requiring reintervention) of 12 % in primary colorectal anastomoses and 9% in anastomoses with a deviating ostomy. (1) In the literature, AL rates after colorectal surgery are reported in the range of 4-20%. (2)

Some aetiological factors of AL are well established. The anastomosis may be poorly constructed, with tension between the afferent and efferent loop, insufficient circulation, or incomplete doughnuts. Healing of the anastomosis may be compromised, as could be the case in patients with diabetes, atherosclerosis or corticosteroid use. However, in many cases, none of these factors seem to explain the occurrence of AL. Therefore, it remains difficult to predict the occurrence of post-operative AL for the individual patient.

Recent studies showed that the composition of the bacterial growth in the intestine influences various processes in the body. For example, bacteria in the intestine are known to influence wound healing, (3) and the intestinal microbiome has recently been linked to the origin of diabetes. (4,5) The development of chemotherapy-induced mucositis is associated with an altered intestinal microbiome. (6) Even the recurrence of Crohn's disease after resection is suggested to be under influence of microbes. (7) There is also a strong suggestion that the composition of the intestinal microbes affects the healing of the anastomosis and might hence be influenced by antibiotics. (8) In addition, selective decontamination of the digestive tract reduces infections and appears to have a beneficial effect on AL in colorectal surgery. (9) However, there are no publications relating the intestinal bacterial growth with surgical outcome of colorectal resections.

We hypothesized that the composition of the intestinal microbiome could play a significant role in anastomotic healing and the occurrence of leakage. The aim of this study was to compare the intestinal microbiota of patients who developed AL with matched patients with healed colorectal anastomoses, without clinical signs of AL.

MATERIALS AND METHODS

Patients

For this study, eight patients who developed AL requiring reintervention were selected and matched with eight patients without AL. Matching was done on gender, age and pre-operative chemotherapy and radiotherapy. All patients were included in the C-seal trial. (2) This multicentre 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. Inclusion criteria were elective colorectal surgery with a circular stapled colorectal anastomosis, age ≥ 18 years, ASA-score <4, mechanical pre-operative bowel preparation and no clinical signs of peritonitis. Exclusion criteria were major surgical or interventional procedures in the 30 days prior to this surgery or other interventional procedures planned within 30 days of entry in this study, and psychological, familial, sociological or geographical conditions which could potentially hamper compliance with the study protocol or the follow-up schedule.

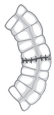
The study was approved by the Medical Ethics Committee of the University Medical Center Groningen – University of Groningen and all participating centres and registered in the Dutch Trial Registry under the number NTR3080. All the patients provided written informed consent. All data were collected anonymously, encoded and saved in a database.

Sample Collection

For all patients who consented to be enrolled in this study we retrieved and stored the 'doughnut'. This 'doughnut' is the small ring of colon and rectum that is cut by the circular stapler to make the anastomosis. Bacterial DNA was isolated from the doughnut and was subsequently analysed using MiSeq sequencing to see whether the microbial composition could be linked with clinical outcome.

DNA Extraction

Total DNA was extracted from 0.25 g of a 'doughnut' using the repeated bead beating method described in detail by Yu and Morrison, (10) with a number of modifications. In brief, four 3 mm instead of 0.5mm glass beads were added during the homogenisation step. Bead beating was performed using a Precellys 24 (Bertin Technologies, Montigny le Bretonneux, France) at 5.5 beats per millisecond in three rounds of 1 min each with 30 s pauses at room temperature in between. The incubation temperature after the bead beating was raised from 70°C to 95°C. Importantly, protein precipitation with 260 µl of ammonium acetate was carried out twice instead of only once. Additional purification steps using columns were not needed after DNA precipitation.



MiSeq Preparation Sequencing Pipeline

The V3-V4 region of the 16S rRNA gene was amplified from the bacterial DNA by polymerase chain reaction (PCR) using modified 341F and 806R primers (Supplementary Table 1) with a 6-nucleotide barcode on the 806R primer as described elsewhere. (11,12) Reaction conditions consisted of an initial 94 °C for 3 min followed by 32 cycles of 94 °C for 45 sec, 50 °C for 60 sec, and 72 °C for 90 sec, and a final extension of 72 °C for 10 min. An agarose gel confirmed the presence of product (band at ~465 base pairs) in successfully amplified samples. The remainder of the PCR product (~45 µl) of each sample was mixed thoroughly with 25 µl Agencourt AMPure XP magnetic beads and was incubated at room temperature for 5 min. Beads were subsequently separated from the solution by placing the tubes in a magnetic bead separator for 2 min. After discarding the cleared solution, the beads were washed twice by resuspending the beads in 200 µl freshly prepared 80% ethanol, incubating the tubes for 30 sec in the magnetic bead separator and subsequently discarding the cleared solution. The pellet was subsequently dried for 15 min and resuspended in 52.5 µl 10 mM Tris HCl pH 8.5 buffer. Fifty microlitres of the cleared-up solution is subsequently transferred to a new tube. The DNA concentration of each sample was determined using a Qubit® 2.0 fluorometer (www.invitrogen.com/qubit) and the remainder of the sample was stored at -20 °C until library normalization. Library normalization was done the day before running samples on the MiSeq by making 2 nM dilutions of each sample. Samples were pooled together by combining 5 µl of each diluted sample. Ten microlitres of the sample pool and 10 µl 0.2 M NaOH were subsequently combined and incubated for 5 min to denature the sample DNA. To this 980 µl of the HT1 buffer from the MiSeq 2x300 kit was subsequently added. A denatured diluted PhiX solution was made by combining 2 µl of a 10 nM PhiX library with 3 µl 10 mM Tris HCl pH 8.5 buffer with 0.1% Tween 20. This 5 µl mixture was mixed with 5 µl 0.2 M NaOH and incubated for 5 min at room temperature. This 10 µl mixture was subsequently mixed with 990 µl HT1 buffer. One hundred and fifty microlitres of the diluted sample pool was combined with 50 µl of the diluted PhiX solution and was further diluted by adding 800 µl HT1 buffer. Six hundred microlitres of the prepared library was loaded into the sample loading reservoir of the MiSeq 2x300 cartridge.

MiSeq Sequencing Pipeline and Statistical Analysis

Software that was used to analyse the data received from Illumina paired-end sequencing, included PANDAseq, (13) QIIME and ARB. (14) Reads with a quality score lower than 0.9 were discarded by PANDAseq. 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 while ARB was

used to identify sequences down to the species level. Principal component analysis (PCA) was performed to describe the variation in all of the bacterial groups into a very limited amount of new relevant dimensions of variability in order to address the issue of multiple testing. In this study only principal component 1, which describes over 67% of the variation in the data, was correlated with the occurrence of AL. The hierarchical clustering analysis was performed with the Hierarchical Clustering Explorer rmed (http://www.cs.umd.edu/hcil/multi-cluster/). The Simpson index was used as a measure of microbial diversity. Nonparametric tests were used, as microbial abundances are rarely normally distributed. Mann-Whitney U or Spearman r tests were used as indicated. The use ± 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

The doughnuts of eight patients with AL and eight patients without AL were analysed. Patient characteristics are listed in Table 1. Body mass index (BMI) was slightly higher in the group of patients with AL, but was not a significant or independent factor for AL in this study group ($P = 0.074$, Mann-Whitney U test).

The microbial composition was successfully determined of 15 of the doughnuts. In one doughnut of a patient in the control group the microbial identification was not successful, probably due to insufficient extraction of bacterial DNA.

Table 1 – Patient Characteristics

	Anastomotic leakage (*n* = 8)	**Control (*n* = 8)**
Gender		
Male	7	7
Female	1	1
Age: min – max (mean) in *years*	57-75 (66.5)	57-75 (66.5)
Surgical indication		
Colorectal cancer	8	7
Diverticulitis	-	1
Preoperative treatment		
Chemotherapy	1	2
Radiotherapy	2	1
Body Mass Index (BMI) (kg/m^2)	30.1	25.4

Bacterial Composition in Relation to AL

The strongest and most straightforward correlation that could be found between the bacterial composition and AL was that the abundance of the *Lachnospiraceae* family (27.3 ± 15.9%) was significantly higher in patients who developed AL as compared to patients who did not develop AL (P = 0.001, Mann-Whitney U test). The most predominant genera of the *Lachnospiraceae* family (*Ruminococcus* (6.1 ± 11.9%), *Blautia* (5.2 ± 4.5%), *Roseburia* (4.4 ± 4.3%) and *Coprococcus* (4.4 ± 4.4%)) all contributed to this particular association. A receiver-operator characteristic (ROC) curve showed that the sensitivity and specificity for the clinical outcome of this finding are high (as can be seen in Supplementary Figure 1). BMI was also positively associated with the abundance of the *Lachnospiraceae* family (P = 0.022, Spearman ρ test).

Complete linkage clustering analysis of the sample on the family level furthermore identified one particular cluster of five samples from patients who developed AL who could be distinguished from the other samples mainly by having the highest *Lachnospiraceae* abundances of all samples. Samples not within this particular cluster (left cluster, Figure 1), had lower *Lachnospiraceae* abundances (P = 0.002, Mann-Whitney U test).

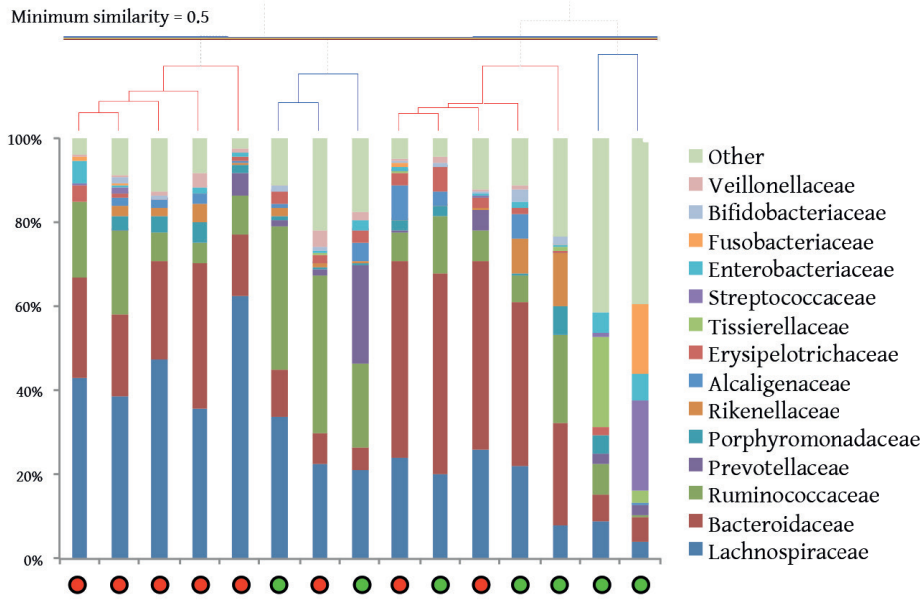


Figure 1 - Hierarchical clustering analysis (top) in combination with the relative abundances of the different microbial families in samples from patients in whom AL occurred (red circles) and of those with no AL (green circles) developed.

Principal component and alpha (a-)diversity analyses on the family level however put the *Lachnospiraceae* association in a slightly different perspective. A principal component analysis shows that principal component 1 (PC1), which accounts for 67% of the variation within the data, is positively correlated with patients developing AL (P = 0.021, Mann-Whitney U test), while the Simpson index, a measure of within (α) sample diversity, is negatively correlated with developing AL (P = 0.037, Mann-Whitney U test). Together, these two variables separate most AL and non-AL patients from one another (Figure 2). Both the Simpson index and PC1 are strongly associated with the abundance of the two most abundant families, *Lachnospiraceae* and *Bacteroidaceae* (23.8 ± 15.5%). The *Lachnospiraceae* abundance is positively correlated with PC1 (P = 0.003, Spearman ρ test) and negatively with the Simpson index (P = 0.007, Spearman ρ test), and the same is true for *Bacteroidaceae* (P = 0.017 and P = 0.015, respectively). As earlier, a ROC curve showed here as well that the sensitivity and specificity of these tests are high, despite the group of only 16 patients (see Supplementary Figure 2).

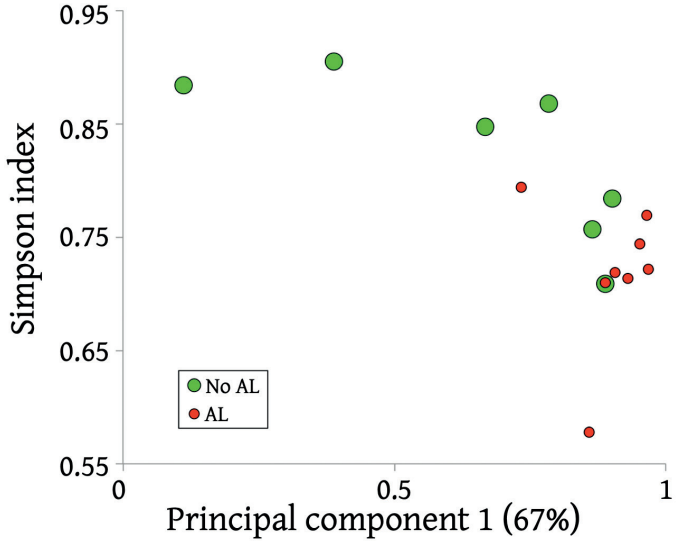


Figure 2 - Principal component analysis (PC1, x-axis) in combination with a diversity analysis (y-axis) in respect to the occurrence (red circles) or absence (green circles) of AL in patients. AL is in general associated with a high score on PC1 and/or a low microbial diversity.

When dissecting the *Lachnospiraceae* finding (37% vs. 17% average abundance, P = 0.001) down to the species level it is found that much of the *Lachnospiraceae* pattern can be attributed to the variation in the amount of *Ruminococcus obeum*, a mucin-degrading bacterium (6.5% vs. 1.7% average abundance, P = 0.021). Mucin-degrading Ruminococci from the *Lachnospiraceae* family as a whole, which include *R. gnavus* and

R. torques, represent the most compelling suggestion for a clinically relevant finding, as their abundance is higher in AL cases (15.5% vs. 3.8% average abundance, P = 0.011). The BMI, however, was only associated (yet not significantly) with *R. obeum* (P = 0.068), but not with the other Ruminococci or with any of the other individual bacterial species.

DISCUSSION

This pilot study on the possible role of the intestinal microbiota in the development of AL after colorectal resection with stapled anastomosis revealed interesting patterns. The correlation that was found between AL and the abundance of *Lachnospiraceae* (Figure 1) was of particular interest as the association between the *Lachnospiraceae* family and AL was unexpected: most of the bacteria from this family are not particularly known to have a negative influence on the bowel. In fact, many butyrate-producing genera are found within the *Lachnospiraceae* family.

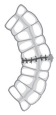
Butyrate is thought to be beneficial as it is the main energy source for colonic epithelial cells. (15) Furthermore, butyrate has been shown to regulate the assembly of tight junctions and to correlate with reduced gut permeability. (16) It also decreases intestinal inflammation by reducing oxidative stress in the colonic mucosa. (17) The *Roseburia* genus in particular is a well-known butyrate producer, which similar to *Faecalibacteria*, is associated with protection against inflammatory bowel diseases. (18) However, a large fraction of the *Lachnospiraceae* reads were identified on the species level to be of mucin-degrading *Lachnospiraceae* (*R. obeum, R. gnavus and R. torques*). (19,20) The abundance of these mucin-degrading bacteria is commonly observed to be elevated in various inflammatory bowel diseases, such as Crohn's disease, Ulcerative Colitis or Irritable Bowel Syndrome. (21-24)

On closer inspection the association between *Lachnospiraceae* and AL could also be the result of the association between obesity and a lower microbial diversity. Obesity is known to be associated with a lower microbial diversity and with a low-grade systemic inflammation. (25-27) In addition, patients with an inflammatory bowel disease are known to have a low microbial diversity in the gut. (27) Beside that, a high BMI is associated with the development of AL. (28-30) Though the number of obese individuals in this study was limited, an association was found between BMI and *Lachnospiraceae* levels. *Lachnospiraceae* levels were strongly negatively correlated with microbial diversity levels that are in turn associated with AL (Figure 2). So, the overabundance of *Lachnospiraceae* (or *Bacteroidaceae* is some cases) might not necessarily be directly linked with the development of AL, but with the absence of other (beneficial) microbial groups.

Alternatively, *Lachnospiraceae* could also be directly linked with AL as an increase in Firmicutes, of which *Lachnospiraceae* are an important member, is commonly found in obese people. (25) Butyric acid is also associated with obesity. (31) While butyrate is commonly associated with many beneficial effects stated earlier, an excess of butyrate might present the body with an excess of energy.

It could also be hypothesized that a poorly diversified microbiome is less stable than a well-diversified microbiome. The administration of prophylactic intravenous antibiotics for instance, as is routinely done in colorectal surgery may cause larger shifts in the bacterial population in a poorly diversified microbiome, offering the opportunity for pathogenic bacteria to repopulate the lumen. The findings of Ohigashi et al. contribute to this theory, as they found that after colorectal surgery the amount of possible pathogenic bacteria, as *Enterobacteriaceae, Enterococcus, Staphylococcus* and *Pseudomonas*, was significantly increased. (32) We do not know, however, whether this also happened during the development of AL in our patients.

The main limitation of this study is the small number of included patients. Beside that, we only investigated AL and obesity in relation to the intestinal microbiota. In a follow-up study, we plan to include a much larger group of C-seal trial patients and perform a more detailed analysis of patient and treatment factors in relation to the intestinal microbiome.



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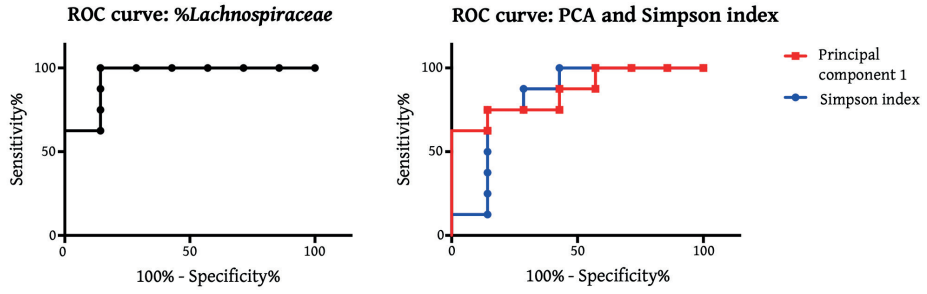
SUPPLEMENTARY FILES

Supplementary Table 1. Nucleotide sequences of primers used in the construction of libraries for Illumina sequencing. Lowercase letters denote adapter sequences necessary for binding to the flowcell, underlined lowercase are binding sites for the Illumina sequencing primers, bold uppercase highlight the index sequences (all the indexes were obtained from Illumina) and regular uppercase are the V3 and V4 region primers (341F on for the forward primer and 806R for the reverse primers). The inclusion of four maximally degenerated bases ("NNNN") maximizes diversity during the first four bases of the run. Diversity is important for identifying unique clusters and base-calling accuracy. (11, 12)

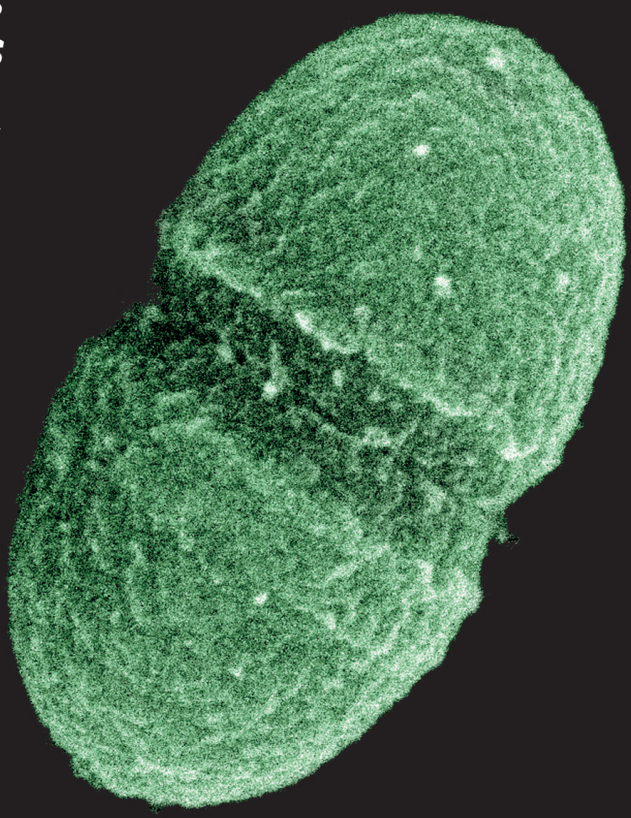
V3_F_ modified	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctNNNNCCTACGGGAGGCAGCAG
V4_1R	caagcagaagacggcatacgagat**CGTGAT**gtgactggagttcagacgtgtgctcttccgatctGGACTACHVGGGTWTCTAAT
V4_2R	caagcagaagacggcatacgagat**ACATCG**gtgactggagttcagacgtgtgctcttccgatctGGACTACHVGGGTWTCTAAT
V4_3R	caagcagaagacggcatacgagat**GCCTAA**gtgactggagttcagacgtgtgctcttccgatctGGACTACHVGGGTWTCTAAT
V4_4R	caagcagaagacggcatacgagat**TGGTCA**gtgactggagttcagacgtgtgctcttccgatctGGACTACHVGGGTWTCTAAT
V4_5R	caagcagaagacggcatacgagat**CACTGT**gtgactggagttcagacgtgtgctcttccgatctGGACTACHVGGGTWTCTAAT
V4_6R	caagcagaagacggcatacgagat**ATTGGC**gtgactggagttcagacgtgtgctcttccgatctGGACTACHVGGGTWTCTAAT
V4_7R	caagcagaagacggcatacgagat**GATCTG**gtgactggagttcagacgtgtgctcttccgatctGGACTACHVGGGTWTCTAAT
V4_8R	caagcagaagacggcatacgagat**TCAAGT**gtgactggagttcagacgtgtgctcttccgatctGGACTACHVGGGTWTCTAAT
V4_9R	caagcagaagacggcatacgagat**CTGATC**gtgactggagttcagacgtgtgctcttccgatctGGACTACHVGGGTWTCTAAT
V4_10R	caagcagaagacggcatacgagat**AAGCTA**gtgactggagttcagacgtgtgctcttccgatctGGACTACHVGGGTWTCTAAT
V4_11R	caagcagaagacggcatacgagat**GTAGCC**gtgactggagttcagacgtgtgctcttccgatctGGACTACHVGGGTWTCTAAT
V4_12R	caagcagaagacggcatacgagat**TACAAG**gtgactggagttcagacgtgtgctcttccgatctGGACTACHVGGGTWTCTAAT
V4_13R	caagcagaagacggcatacgagat**CGTACT**gtgactggagttcagacgtgtgctcttccgatctGGACTACHVGGGTWTCTAAT
V4_14R	caagcagaagacggcatacgagat**GACTGA**gtgactggagttcagacgtgtgctcttccgatctGGACTACHVGGGTWTCTAAT
V4_15R	caagcagaagacggcatacgagat**GCTCAA**gtgactggagttcagacgtgtgctcttccgatctGGACTACHVGGGTWTCTAAT
V4_16R	caagcagaagacggcatacgagat**TCGCTT**gtgactggagttcagacgtgtgctcttccgatctGGACTACHVGGGTWTCTAAT

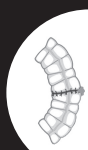


Supplementary Figures 1 & 2 – Receiver-operator characteristic (ROC) showing the sensitivity and specificity for the clinical outcome of the percentage of *Lachnospiraceae* and the diversity indices PCA and Simpson index.

ENTEROCOCCUS FAECALIS

E. faecalis is a Gram-positive coccus and a facultative anaerobic commensal bacterium living in the human gastrointestinal tract. It is known to cause life-threatening infections and has a high level of antibiotic resistance. This species is associated with anastomotic leakage and is amongst others discussed in Chapter 7 of this thesis.





CHAPTER 3A

MUCUS MICROBIOME OF ANASTOMOTIC TISSUE DURING SURGERY HAS PREDICTIVE VALUE FOR COLORECTAL ANASTOMOTIC LEAKAGE

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ABSTRACT

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 "doughnuts" 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.

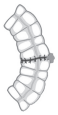
Results

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.

INTRODUCTION



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 defence 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 diarrhoea. (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 appears 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 analyse 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 gender, 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 multicentre 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.

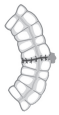
The study was approved by the Medical Ethics Committee of the University Medical Center Groningen and all participating centres. The trial was registered in The Netherlands National Trial Register under the number NTR3080. In total 539 patients were included, all patients provided written informed consent; 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 analysed 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 analyse 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 (FISH) 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 'doughnut'. 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-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 aatgatacggcgacca ccgagatctacactctttccctacacgacgctcttccgatctNNNNCCTACGGGAGGCAGCAG & caagcagaagacggcatacgagat**CGTGAT**gtgactggagttcagacgtgtgctcttccgatct GGACTACHVGGGTWTCTAAT 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-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 clean-up, and MiSeq library preparation, as described by Heida et al. (17), are found in Supplementary Data File 1.

MiSeq Sequencing Pipeline and Statistical Analysis

Software that was used to analyse the data received from Illumina paired-end sequencing, included PANDAseq, (18) QIIME and ARB. (19) Reads with a quality score lower than 0.9 were discarded by PANDAseq. 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 in order 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 ± 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 one 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 non-microbiota 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.

Table 1 – Patient Characteristics

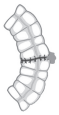
	AL (n = 29)	No AL (n = 94)	P-value
C-seal / No C-seal	18 / 11	45 / 49	0.207
Gender male / female	22 / 7	61 / 23	0.365
Age years (sd)	63.4 (10.4)	63.4 (10.4)	0.510
Body Mass Index (sd) kg/m^2	26.0 (3.9)	26.9 (4.0)	0.075
Charlson Comorbidity Index			0.415
0	16	56	0.830
1	5	23	0.462
2+	8	15	0.179
Indication 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

Surprisingly almost no difference was found between AL and non-AL patients when the 118 samples were analysed 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.



Principal Component & 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 (Figure 1). This highlights some of the main differences between C-seal patients and non-C-seal patients in 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, while the other three 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 appear 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 which 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%) (Supplementary Figure 1). 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 (Figure 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 *Prevotella 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 (Table 2). *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).

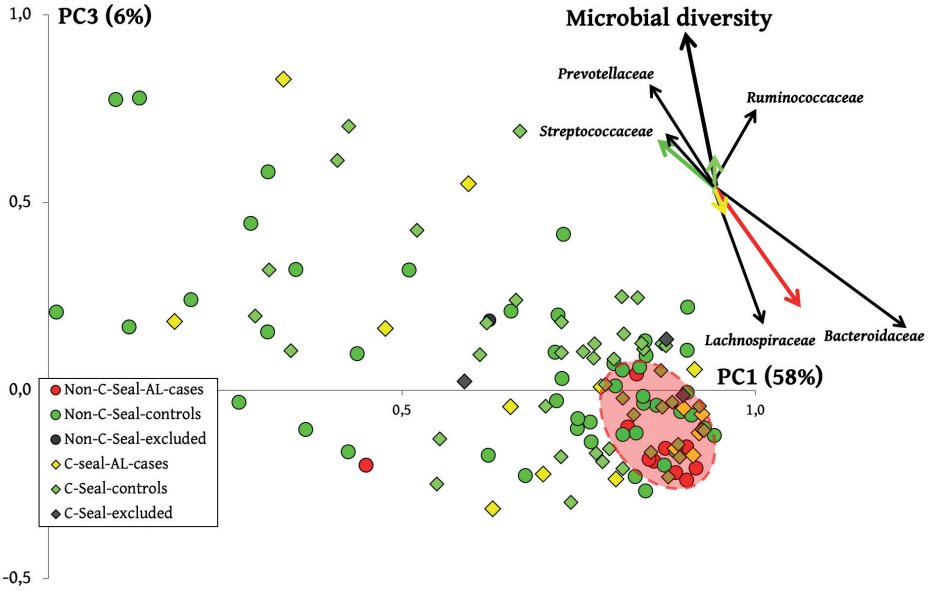


Figure 1 - Principal component analysis (PCA) plot of all 122 samples, divided over all 4 groups and relevant associations. PC1, represented by the x-axis, is associated with AL in non-C-seal patients and describes 58% of the variation in the data. PC3, represented by the y-axis, is positively associated 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. Coloured 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*.

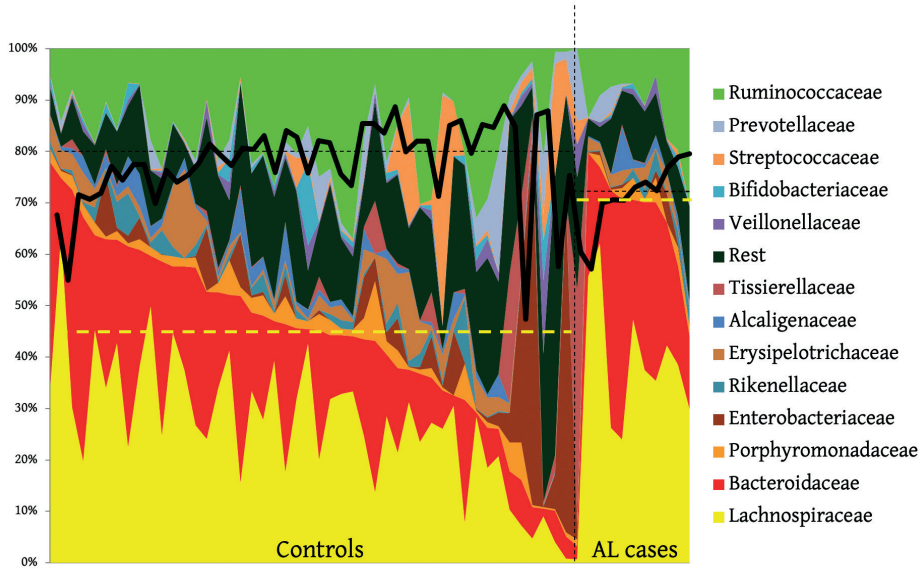


Figure 2 - Microbial composition profile of non-C-seal patients. AL cases are depicted to the right of the dashed vertical line and controls are on its left. The dashed horizontal black lines represent the median Simpson index value (%/100) of controls (left) and AL cases (right) and show that the diversity is higher in controls (P = 0.002). The orange lines represent the median values of the sums (%) of the two 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)).

Table 2 - Main associations with AL in non-C-Seal samples (MW-U test)

Reduced risk	**Average**	**P-value**
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

Predictive Analyses

As Figure 1 and Supplementary Figure 1 show, AL cases of non-C-seal patients appear 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 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 lower than 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).

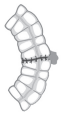
DISCUSSION

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 analysed 16 non-C-seal patients of whom 8 developed AL. (13) The current study included an additional 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 two families appears 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- 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, e.g. *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 appears 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 three most dominant microbial families in the gut, *Bacteroidaceae* (19%), *Lachnospiraceae* (29%) and *Ruminococcaceae* (16%), the first two 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 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 affect 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 *Prevotella 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 faecal 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 non-pathogenic *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. Since this is very difficult, alternatively, fecal samples could be collected both before and after surgery, in addition to the "doughnut" sample taken during surgery.

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

CONCLUSION

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 prior to 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.

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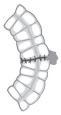

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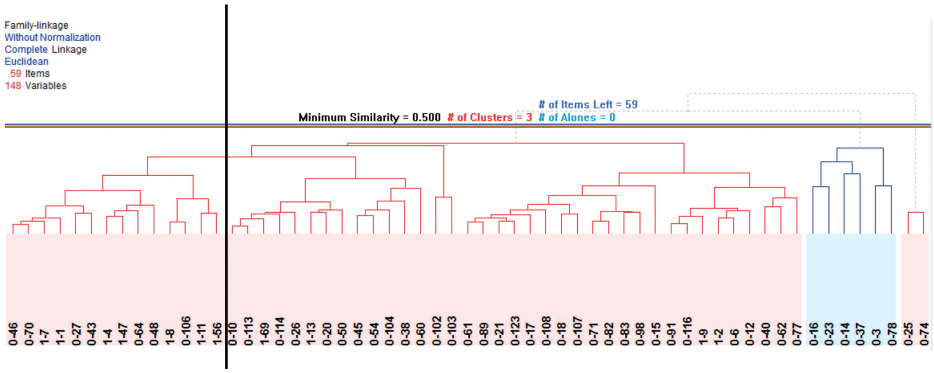
SUPPLEMENTARY FILES

Supplementary Data File 1

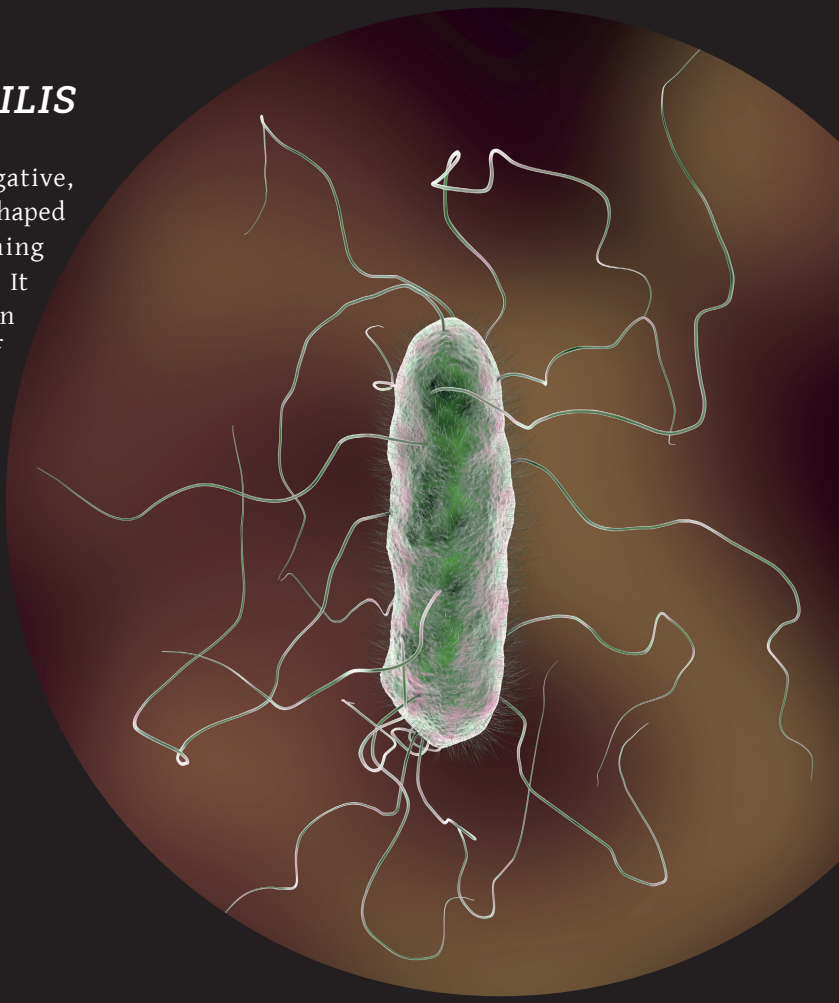
Reaction conditions consisted of an initial 94 °C for 3 min followed by 32 cycles of 94°C for 45s, 50°C for 60s and 72°C for 90 s, and a final extension of 72 °C for 10 min. An agarose gel confirmed the presence of product (band at ~465 base pairs) in successfully amplified samples. The remainder of the PCR product (~45 µl) of each sample was mixed thoroughly with 25 µl Agencourt AMPure XP magnetic beads and was incubated at room temperature for 5 min. Beads were subsequently separated from the solution by placing the tubes in a magnetic bead separator for 2 min. After discarding the cleared solution, the beads were washed twice by resuspending the beads in 200 µl freshly prepared 80 % ethanol, incubating the tubes for 30 s in the magnetic bead separator and subsequently discarding the cleared solution. The pellet was subsequently dried for 15 min and resuspended in 52.5 µl 10 mM Tris HCl pH 8.5 buffer. Fifty microliters of the cleared-up solution is subsequently transferred to a new tube. The DNA concentration of each sample was determined using a Qubit® 2.0 fluorometer (www.invitrogen.com/qubit), and the remainder of the sample was stored at -20 °C until library normalization. Library normalization was done the day before running samples on the MiSeq by making 2 nM dilutions of each sample. Samples were pooled together by combining 5 µl of each diluted sample. Ten microliters of the sample pool and 10 µl 0.2 M NaOH were subsequently combined and incubated for 5 min to denature the sample DNA. To this, 980 µl of the HT1 buffer from the MiSeq 2x300 kit was subsequently added. A denatured diluted PhiX solution was made by combining 2 µl of a 10 nM PhiX library with 3 µl 10 mM Tris HCl pH 8.5 buffer with 0.1 % Tween 20. This 5 µl mixture was mixed with 5 µl 0.2 M NaOH and incubated for 5 min at room temperature. This 10 µl mixture was subsequently mixed with 990 µl HT1 buffer. One hundred and fifty microliters of the diluted sample pool was combined with 50 µl of the diluted PhiX solution and was further diluted by adding 800 µl HT1 buffer. Six hundred microliters of the prepared library was loaded into the sample loading reservoir of the MiSeq 2x300 cartridge.

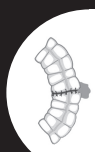


Supplementary Figure 1 – Hierarchical clustering analysis. Fifty percent of the samples in the cluster left of the black line are AL (1) (7 of 11 AL samples). Only 9% of samples that are not in the left cluster (but to right of the line) are AL. Thus, AL samples are associated significantly more often with the microbiota composition represented by the cluster left of the line (*Lachnospiraceae* dominated): Chi-square test: P = 0.0006.

PROTEUS MIRABILIS

P. mirabilis is a Gram-negative, facultatively anaerobic, rod-shaped bacterium. It has swarming motility due to its flagella. It is predominantly found in the gastrointestinal tract of humans. It is mostly noted for infections in the (catheterized) urinary tract, but is capable of causing a variety of infections. This species is associated with anastomotic leakage and is amongst others discussed in Chapter 7 of this thesis.





CHAPTER 3B

RESPONSE TO COMMENT ON "MUCUS MICROBIOME OF ANASTOMOTIC TISSUE DURING SURGERY HAS PREDICTIVE VALUE FOR COLORECTAL ANASTOMOTIC LEAKAGE"

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RESPONSE TO COMMENT

This letter is in response to the letter by Lamendella and Stewart, which argued the used statistics in our paper (1).

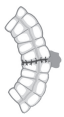
The first point made by the authors is that we selected two cohorts of patients, with no difference in anastomotic leakage between the groups, and that we made an inappropriate subgroup analysis as is common in 'the genre of microbiome causing leaks'. Aside from the fact that literature that focuses on the microbiome causing anastomotic leaks is still sparse, the point itself is incorrect.

The microbiome analyses we performed were done on circular stapler donuts that were collected during the C-seal trial. This trial was designed to evaluate the efficacy of the C-seal, a biodegradable intraluminal sheath, in avoiding clinically relevant anastomotic leakage. Unfortunately, this trial was discontinued after interim analysis due to futility (2). A subset of patients consented to use the donut for further analyses. Based on availability, we could use the donut of 29 patients who developed anastomotic leakage, which we matched with 94 patients who did not develop anastomotic leakage based on sex, age, and preoperative chemotherapy and radiotherapy, all stated in our article. Although we did not match on the use of the C-seal, it was to be expected that the percentage of C-seal patients in the selected group is roughly 50% as the original randomization was done on a one to one basis.

Interestingly, when we compared the microbiome of the 29 patients with a leak with the 94 without a leak, there was only a slight difference - the *Blautia* genus was more abundant among AL patients (P = 0.040), see also our article - , contrary to our pilot study of 16 patients (all without the C-seal). (3) A subsequent analysis of C-seal and no C-seal patients was not more than logical, with the additional benefit that the microbiome might help explain the results of the C-seal trial (see Discussion).

Since there were no truly convincing differences found between C-seal AL-cases and controls, whereas the opposite was found for the non-C-seal cohort (and thus the regular procedure), one can conclude that the presence of a C-seal might have influenced the microbiota severely after application. However, this then occurred after our sampling and is not seen in our analysis.

We accept that improvements can indeed be made to the 16S data processing pipelines, yet these would not have altered the main findings of this work. A stricter quality score filter would indeed cause fewer low-quality reads to inflate the apparent diversity or the total number of observed species. We have as such specifically chosen not to use rarefaction curves; we furthermore mainly focus on the dominant species present within the samples as they determine most of the metabolic (and other) activity. We do this by utilizing the Simpson diversity index and Principal Component Analysis to analyse our data, both techniques that focus on the abundant groups. Our



main findings, in which we have confidence, are in regards to the more prevalent bacterial groups, not the rare ones (which might have been missed).

Species level identification might have been thought to be difficult in 2009 (see reference from authors Letter to the Editor) but it can be done for most species now, using 16S reads covering multiple variable regions (V3 and V4), as reference libraries have increased in size and quality. We would now however recommend a different method of taxonomic assignment as utilized in this study, which is called oligotyping. (4) Instead of allocating reads to the closest previously defined taxonomic box of a particular species, oligotyping instead combines all the reads of a study into its own set of taxonomic boxes (oligotypes). A representative sequence of each oligotype can then be compared to the reference library, frequently even allowing for the identification of different strains/oligotypes of the same species (Supplemental Figure 4 of Salter et al, 2017(5)). Aside from this, the main findings in our study were on the family level, using the species level just as additional detail.

Regarding the *Lachnospiraceae* family and butyrate production; it indeed contains various important butyrate producers such as the *Roseburia* and *Coprococcus* genus. This family however also contains dozens of non-butyrate producing species. This is one of the reasons why the genus and species level were also analysed. It was found that within the *Lachnospiraceae* family that the non-butyrate producing species accounted for most of the difference between non-C-seal cases and controls. Somewhat elevated levels of *Roseburia* and *Coprococcus* were indeed observed in cases, but not in a significant manner. The *Ruminococcaceae* family, which includes the (more) important butyrate producer *Faecalibacterium prausnitzii*, was associated with an increased diversity of the gut microbiome.

Principal component analysis, an unsupervised dimension reduction method (SPSS standard settings, no rotation), is used to show both visually and statistically that a significant difference does exist between cases and controls of a particular multidimensional dataset. Principal component 1, which describes more than half of all the variation in the data (58%), clearly demonstrates that non-C-seal AL cases score significantly higher on PC1 than non-C-seal controls. This shows that a particular gut microbiota composition is significantly associated with anastomotic leakage. The next step is to ascertain what this difference, as described by PC1, exactly is. PC3, which describes 6% of the variation was also associated with AL-status in non-C-seal cases and was similarly of interest. Correlation analyses with these particular principal components can subsequently be utilized to identify which combination of species is driving the mucus microbiome to be more prone to AL development.

In order to use PCA in such a fashion, the data indeed need to be normalized in such a way that sampling depth is simply irrelevant (at least for the somewhat abundant

bacterial groups). To do so all bacterial groups are normalized into a percentage of reads per sample, as for example shown in Figure 2, before being further analysed. We assumed (apparently incorrectly) that this was self-evident. We acknowledge that the predictive analysis has an element of ad hoc semblance, yet any set of parameters focusing on diversity and on the two main bacterial families, without the focus on equal numbers, would have resulted in the same conclusions.

The main weakness of this set of parameters is not the statistical aspect but the potential universal applicability aspect. If samples for example in future studies are stored in slightly different conditions, for a longer or shorter time, being processed with different reagents (different DNA isolation kit), etc. then the percentages of different bacterial groups after sequencing may differ. Furthermore, the gut/mucus microbiome differs between inhabitants from different countries. These parameters can thus not be used yet to make decisions on whether to treat a patient or not. They do however possibly provide us with a "direction" in regards where to steer the gut microbiota composition towards in patients. Basically, eat more fibre and vitamins (vegetables) and less sugar, (animal) protein and (saturated) fats.

As discussed above, we still support our study and defend its conclusions. We believe our statistics and methods for an exploratory study of a limited and unique set of samples were appropriate and therefore academically meaningful. We hope the academic world will engage in more studies into the relation of the mucus microbiome and anastomotic leakage, as the aetiology of anastomotic leakage is still unclear in many cases.

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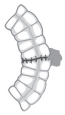

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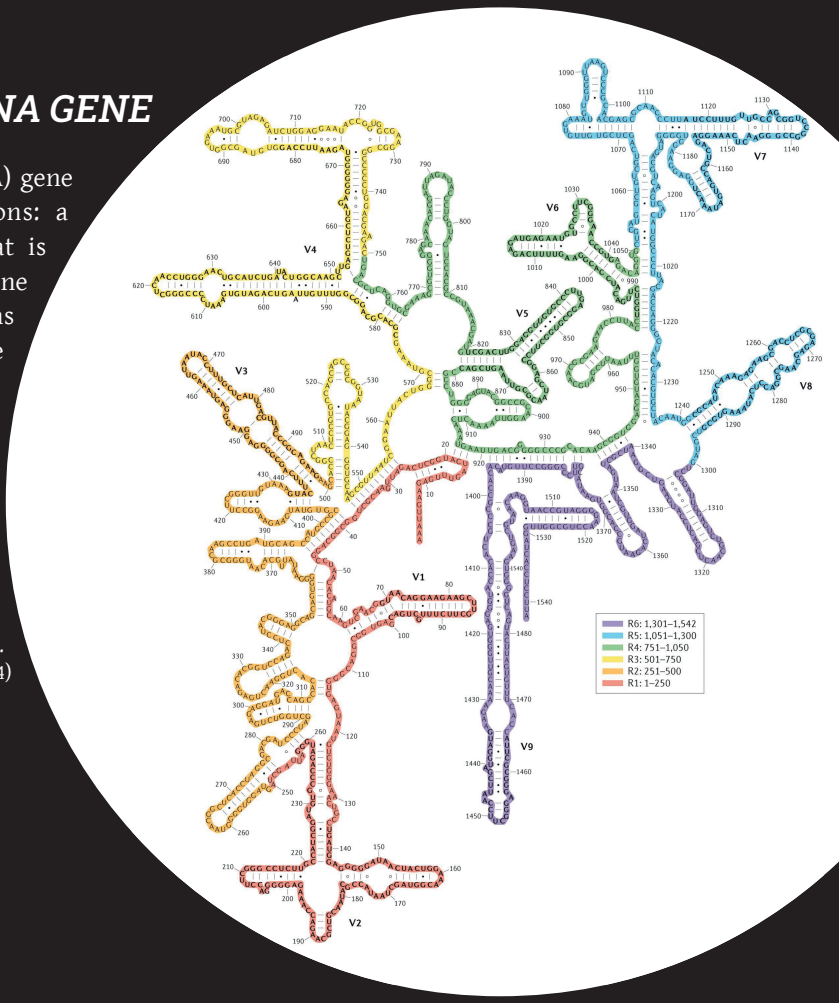
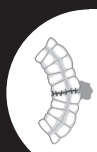
16S RIBOSOMAL RNA GENE

The 16S ribosomal RNA (rRNA) gene contains two sorts of regions: a highly conserved region that is present in all bacteria and nine separate hypervariable regions (V1-V9) that are specific to the taxonomic rank of the microbe. This molecular barcode can therefore be used to identify the different bacteria.

Source: Yarza, P., Yilmaz, P., Pruesse, E. *et al.* Uniting the classification of cultured and uncultured bacteria and archaea using 16S rRNA gene sequences. *Nat Rev Microbiol* **12**, 635–645 (2014)





CHAPTER 4

MICROBIOME RESEARCH: A PRIMER FOR THE INTERESTED SURGEON

Submitted

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ABSTRACT

Objective

The aim of this review is to inform the interested surgeon about the culture-independent microbiome research.

Observations

Over the last two decades, we have come to understand the important role of the human microbiota, a vast ecosystem encompassing all of the bacteria, fungi, archaea, viruses, and protozoa colonizing the human body. Extensive research has demonstrated the importance of the microbiota living in and on the human body are major players in health and disease. Research on the microbiota and their genes, the so-called microbiome, have become widely accepted, but publications in the surgical field remain scarce and have fallen behind on other medical specialties. We think this might be due to a lack of knowledge about the possibilities and methods. Therefore, we wrote this review that explains the current methods and considerations for performing microbiome research and the interpretation of said research.

Conclusion

Microbiome research in the surgical field is rare and it is time to gap to bridge. Current methods are not perfect yet to analyse or interpret microbial data. There is no universal protocol, there are very diverse methods, that can be interpreted differently. This review gives a practical overview on the possibilities and pitfalls in microbiome research. When one takes the subjects discussed in this review in consideration, conducting 16S rRNA gene sequencing can be a standard method for microbial research that surgeons should consider.

INTRODUCTION

Over the last two decades, we have come to realize that the human microbiome, a vast ecosystem encompassing all of the bacteria, fungi, archaea, viruses, and protozoa colonizing the human body, plays an important role in human health. The advent of culture-independent techniques to analyse the microbiome, such as 16S ribosomal RNA gene or metagenomic DNA sequencing, has led us to discover that the variety of organisms inhabiting the human host is wider than ever imagined. Further, it is now appreciated that the genetic composition of these microorganisms and their interaction with human genes are critical to regulate host homeostasis and the balance of health and disease.

Knowledge on the influence of the microbiome on medical diseases has quickly advanced and has touched all medical subspecialties. There is now compelling evidence that diseases such as cancer, depression and even multiple sclerosis are at least in part microbial driven and bacterial derived therapies such as faecal transplantation for *Clostridium difficile* infections or probiotics for inflammatory bowel disease are becoming common. (1-4) While specialized investigators have linked intestinal anastomotic leak, postoperative ileus, trauma outcomes to perturbations of the microbiome, how the microbiome influences surgical patients remains at its infancy. (5-8) It is therefore not surprising that there are significantly more manuscripts on microbial science published in the top medical compared to the equivalent surgical journals (Figure 1).

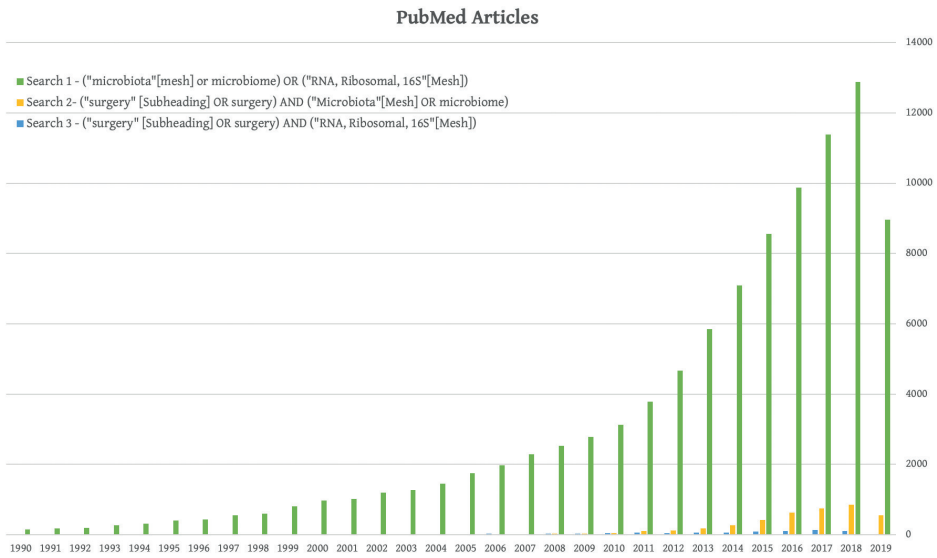


Figure 1A – Search results on PubMed. Including the search term "*surgery*" in simple PubMed searches, a rise in microbiota related research is seen. However, a general search for research articles on microbiota shows that the surgical contribution to these articles is minor. Search terms used are indicated in the legend of the graph.

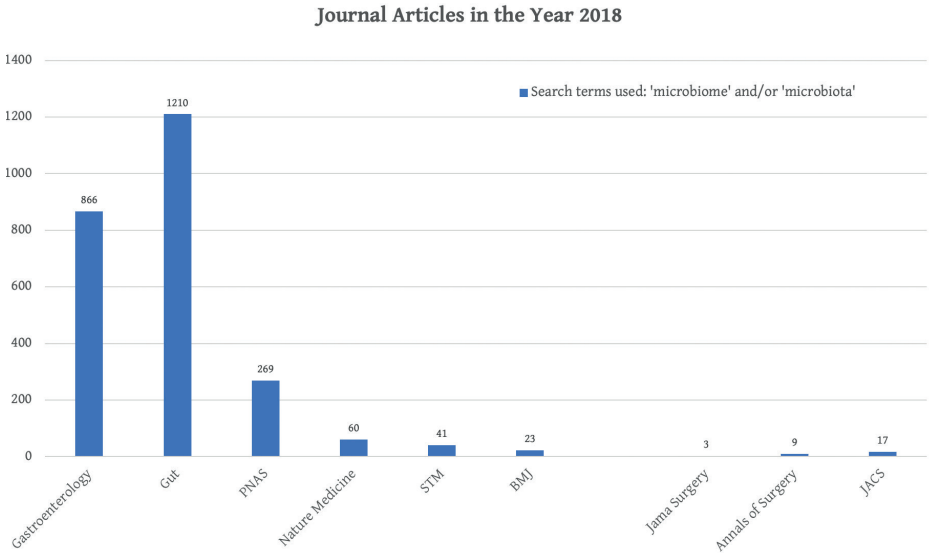
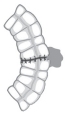


Figure 1B – Search results on various journals' websites. A search on the websites of several well-known journals with the terms: "*microbiome*" and/or "*microbiota*" shows that the surgical journals publish considerably less articles containing research on microbiota than journals with non-surgical scopes.

Although it is clear that surgeons are intrigued by the microbiome, most do not have training in the innovative techniques that are driving microbial research. This gap in knowledge represents a major hurdle for surgeons to have widespread participation in the field and potentially prevents the advance of our understanding as to how the microbiome influences surgical patients. In this manuscript, we review the tools and methods of microbiome research, giving a foundation for the interested surgeon to develop their own microbial research program or to critical evaluate the existing microbial based literature.

BACTERIA AND THE HOST

The work done over the last decades has demonstrated the importance of intestinal bacteria in health and disease. Intestinal bacteria are in physiological condition in complete symbiosis with their host. They provide nutrition for epithelial cells by production of short chain fatty acids (SCFAs). These are produced by microbial fermentation of dietary fibres and endogenous mucus, in turn continuously produced by goblet cells to maintain the mucus barrier. Host and bacteria are in continuous contact and exchange not only nutrition but also information. Microbiota stimulate epithelial turnover and help regulating the host's immune system in order to maintain the bacterial composition and prevent and pathogenic colonization. (9,10) Additionally,



bacterial signalling has been proven to contribute to epithelial cell wall integrity. (11) Besides, bacteria that are potentially pathogenic such as *Pseudomonas*, *Enterococcus Staphylococcus* and *Enterobacteriaceae* like *Escherichia* and *Klebsiella*, are usually also present in small quantities in the non-diseased gastrointestinal tract. However, during ilness the bacterial composition is changed or changes. Much research of the microbial composition and intestinal diseases such as IBD and IBS have been conducted. (12) Even in the youngest the microbiota are linked to a role in disease, where microbial analysis of the meconium shows a relationship with necrotizing enterocolitis. (13,14) Also postoperative surgical infections are associated with a disturbed microbiome. (15) Research from our own laboratories also show relationship with the microbiota and anastomotic leak in colorectal surgery. (5,16) Another interesting example of microbiome analysis with next generation sequencing is an association between tongue microbiota and liver carcinoma. It shows that patients with liver carcinoma have a significantly distinguishable microbiome on the tongue compared to healthy patients. (17)

BASICS OF BACTERIAL RESEARCH

The verbiage used for microbial research has evolved as the field has matured. The *Microbiome* refers to the collective genomes of the microorganisms in a particular environment, while the *microbiota* refers to the specific microorganisms that are found within a particular environment. It is important to recognize that while most often the researcher is studying the bacterial component of the microbiota, other microorganisms, such as fungi and viruses are gaining increasing importance of their contribution to the role of the microbiota in health. For simplicity, for the remainder of this manuscript, we will refer to only the bacterial component of the microbiota.

Bacterial Identification

Culture-dependent methods

The initial question asked in most microbial centred research projects is '*which bacteria are present*?' Culture-dependent identification of bacteria has been the standard for nearly two-centuries. In this technique, a biological sample is inoculated on general or selective media leading to the identification of the bacteria in question. Detection of pathogens can be done with a very low limit of detection, one growing bacterium in a sample will lead to a colony on a plate. The bacterial strains can then be stored for further testing. Because this method is dependent upon bacterial growth, the live strain can be exposed to different environments and the physiological function assayed. Additionally, experiments can be designed to silence or overexpress bacterial

genes to understand the molecular mechanisms of the pathogens. Culture-dependent cultivation of bacteria remains the standard of care for patient derived pathogen identification and antibiotic resistance profiling in medical laboratories across the world.

While culture-dependent identification of bacteria has been the cornerstone for bacterial analysis since its discovery, its results are entirely reliant upon that ability of the tested microbe to grow *in vitro*. Oxygen concentration (aerobes vs anaerobes) and nutrient requirements vary for each species, and thus if they are not optimized that strain will not grow or be identified if present.

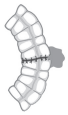
Culture-independent methods

The process by which culture-independent procedures are performed is significantly different from culture-dependent methods. Whereas culture-dependent methods rely on growth of the organism in the laboratory, culture-independent techniques depend solely upon the number of molecules that represent a micro-organism. This could be proteins and fatty acids but most of the times these are nucleic acids present in the sample being tested. Most techniques are based on the detection of the ribosome, for instance 16S rRNA that is the RNA portion of the small subunit of the ribosome in prokaryotes (bacteria and archaea) and that is significantly different from the homologue 18S rRNA molecule present in eukaryotes (e.g. human). Unique to prokaryotes, the 16S rRNA gene contains two sorts of regions: a highly conserved regions present in all bacteria and 9 separate hypervariable regions that are specific to the taxonomic rank of the microbe. This molecular barcode can therefore be used to identify which bacteria are present within a biological sample.

In a standard workflow, the biological sample is acquired (detailed below), and DNA is extracted. Using primers that bind to both the conserved regions and that flank the hypervariable regions, the 16S rRNA gene is amplified. (18) This gene segment (known as an amplicon), is subsequently sequenced, and compared to 16S rRNA genes in reference databases. This comparison then allows identification of the bacteria for which the 16S rRNA gene was amplified from.

rRNA or rDNA?

First the terminology has to be addressed that in literature 16S rRNA sequencing, 16S rDNA sequencing or 16S rRNA gene sequencing are used, sometimes even for the same analysis. They are in fact not the same. Ribosomal RNA (rRNA) is the actual RNA product of transcription from the ribosomal DNA (rDNA operon). rRNA is quite stable and RNA-based 16S rRNA sequencing allows differentiation between bacteria that are metabolically active or in a dormant state, since the number of ribosomes



is dependent on the growth state. In order to PCR amplify and sequence this, first a reverse transcriptase reaction will convert the RNA into cDNA. In contrast, DNA-based 16S sequencing (rDNA) (also called 16S rRNA gene sequencing) is unable to differentiate between alive and dead bacteria. (19) However, the latter method is the most general used in the research setting. We should thus speak about 16S rDNA sequencing or better 16S rRNA gene sequencing, since the sequence of this ribosomal RNA gene is the actual product that is sequenced.

DETAILS ON PERFORMING CULTURE-INDEPENDENT BACTERIAL IDENTIFICATION

To conduct culture-independent microbiome analysis, the sample must first be acquired and stored. Next, the bacterial DNA is extracted and sequenced. Finally, the sequenced DNA is analysed to determine bacterial composition. In this section, we highlight the methods and techniques of each step.

Sample Collection

Type of biological sample

There is a multitude of biological samples that can be analysed for bacterial composition and in the human microbiome project, 18 body sites were analysed. (20) Depending upon the goals of the project, researchers may focus on a single location for sample acquisition, or obtain samples from multiple. When obtaining samples, to limit contamination, samples should only be touched with sterile instruments, and placed into sterile containers. (21) For skin or mucosal samples, most researchers will use a specialized swab (e.g. FLOQSwab®) which includes a preservation medium (e.g. eNAT®). To collect the sample, the swab should be rubbed on the location for a consistent amount of time. For faecal samples, stool can simply be collected with a sterile container.

Because most studies focus on the gut microbiome, the most commonly used samples for microbiota analysis are faecal samples. These samples are relatively easily obtained, and many consider them representative for the intestinal microbial composition. (22) While a faecal sample may represent a sampling of the entire gut microbiome, it has been clearly shown that the bacterial composition changes throughout transit through the GI tract. (23) Investigators have observed normal spatial compositional changes between the small bowel, colon and rectum, and between luminal and mucosa adhered samples. (23,24) A further consideration when obtaining GI-tract samples is the difference between expelled stool and intraluminal contents. While most faecal samples will be expelled stool self-collected by the patient,

surgical researchers may be interested in analysing intra-luminal stool from a surgical resection or at an anastomosis. Similarly to differences throughout the gut there is a significant difference between composition in bacteria that colonizes the mucosa and which exists in the faeces. (25) A final consideration, especially for patients undergoing surgery, is the perioperative prescription of an oral mechanical bowel preparation. Mechanical bowel preparation can significantly alter the intestinal microbiome and increase the impact of oral antibiotics on the intestinal, and in particular colonic, mucosa. Interestingly, this influence is not universal and is variable between healthy and inflamed bowels. (26,27)

Regardless of the location for which the sample is acquired, it is critical that during analysis similar samples must be compared. For example, mucosa samples should be compared to mucosa samples, while expelled stool can be compared to expelled stool. If surgical patients are being studied, prescription of a bowel prep should be noted, and samples consistently be collected either before or after, depending upon the study aims.

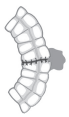
Sample storage

Once the sample is obtained, it can be stored indefinitely if stored properly. The method by which the sample is stored can significantly influence the outcome of the microbial analysis and thus all samples should be stored in the same manner. Stabilization of DNA is essential so that the analysis represents the conditions when the sample was acquired. Collected samples should be transported on ice and stored at either –20 °C or, ideally, at –80 °C. (28) If stored at –80 °C the samples can be stored indefinitely. Due to logistics, temporarily storing the samples at 4°C is often necessary and if less than 24 hours, this should not significantly influence the bacteria composition; in this case the sample should be placed at -80°C as soon as possible. (29)

While this method of storage allows non-culture based analysis, it does not allow subsequent direct culture of the bacteria. If direct culture is also to be performed, the sample should be aliquoted mixed with sterile glycerol-physiologic salt solution (10-25%). Glycerol will prevent crystal formation and thus will preserve the sample to allow for subsequent bacterial culture. (30)

DNA isolation and amplification

The first step in performing 16S rRNA gene bacterial analysis is isolation of bacterial DNA. Isolation of genomic DNA is a standard laboratory procedure but fraught with many pitfalls and influencing factors. Isolation of bacterial DNA can be outsourced at many ‘core-facilities’ or performed by the investigator using almost any of the commercially available DNA-isolation kits. These kits rely on different principles



for DNA purification, including solution- and solid-phase-based protocols and the majority of available kits have satisfactory sequencing results (31,32) However, the yield and quality of the DNA-isolates and the community structure observed from various kits are not always comparable or interchangeable. (33) Therefore the same kit, and preferable with the same lot number, should be used for a single study. (32,34-36). Polymerase chain reaction (PCR) amplification of DNA is done by first denaturizing every double-stranded DNA copy present and creating two single stranded complement copies. By adding matching nucleotides (polymerizing) the two single-stranded copies become double-stranded again. Repeated denaturization and polymerization, causes an exponential amplification of the amount of DNA copies. It is important to remember that any extraction and amplification kits contain their own microbiome, therefore, contamination can influence the results. This is especially important when the sample material has low biomass, increasing the relative amount of the contaminant, which in the worst cases, results in "blue whales in the Himalaya or African elephants on Antarctica". (21)

DNA sequencing

While there are different methods of sequencing the most widely used is the Illumina MiSeq sequencing method and has become highly accurate and relatively affordable. It can be used for both single-end and paired-end sequencing. Paired-end sequencing reads from both ends of the isolated DNA fragments, allowing to detect the distance between each paired read due to overlapping base-pairs. This results in double the length of single-end sequencing reads and is therefore more accurate for phylogenetic identification. Single-end sequencing is more affordable, but only reads the DNA from one end. It is therefore less accurate, especially for the complex mixtures of 16S rRNA genes, and not advisable for microbiome research. It is often used for RNA-sequencing (transcriptomic analysis).

In addition to Illumina MiSeq, novel methods have recently become available allowing sequencing to become more mainstream. The MinION Nanopore system is small, lightweight, and can be plugged into a desktop computer without the need of additional computing infrastructure required. (37) The Ion Torrent is a very efficient methods and sequence runs are very fast (about 2 hours compared to the 5.5 to 60 hours for Illumina). It is relatively low-cost and has been able to rapidly expand the output in a short time. (38) Despite these advances, laboratory equipment and preparation are needed for the preparation steps limiting its widespread use.

METHODS OF ANALYSIS

Analysis of the sequenced bacterial DNA is the next step. In most academic centres, bioinformatics is available for help in guiding analysis. In many instances, bioinformatics centres will provide complete analysis and figure generations. In this section we review the available methods used for analysis that allow the surgeon critical evaluate the results.

Taxonomic analysis

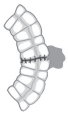
Taxonomic analysis is performed to determine which bacterial taxa the sequenced DNA belongs to. To do this, the sequenced hypervariable regions of the bacterial 16S gene are used. This gene contains nine hypervariable regions (V1-V9) ranging from 30-100 base pairs. It has been shown that the V4-V6 regions are the most reliable regions for representing the full-length 16S rRNA gene sequences in the phylogenetic analysis of most bacterial phyla, while V2 and V8 were the least reliable regions. (39) Therefore, amplification of the V4 region is the most commonly used method for taxonomic analysis.

To determine which taxa the amplicon originated from, the sequenced variable region is aligned to an available reference database such as RDP, (40) SILVA, (41) NCBI, (42) Greengenes (43) and the more recently published Open Tree of life Taxonomy (OTT). (44,45).

Taxonomy analysis can classify the amplicon into one of seven taxonomic ranks (Table 1). To which level taxonomic analysis can identify depends upon the length of the amplicons: a longer sequence gives a better and more accurate identification. Currently often only the V4 region of the 16S rRNA gene is sequenced, while sequencing V3 and V4 by using a different primer set for amplicon generation, gives more information. In the literature, the most commonly used representations are phylum and genus level but the choice of rank in which the authors report their results differs per experiment and on the hypothesis being tested.

Table 1 – Example of scientific classification of the taxonomic ranks for the well-known bacteria *Escherichia coli* and the domestic cat.

Domain/Kingdom	**Bacteria**	**Animalia**
Phylum	Proteobacteria	Chordata
Class	Gammaproteobacteria	Mammalia
Order	Enterobacteriales	Carnivora
Family	*Enterobacteriaceae*	Felidae (subfamily Felinae)
Genus	*Escherichia*	*Felis*
Species	*E. coli*	*F. catus (domestic cat)*



Relative Abundance

Once the taxa of all of the amplicons within a sample are determined calculating the relative abundance is often the next step in analysis. Relative abundance shows the percentage of an organism within the total number of organisms present in that sample. This analysis is the simplest method to compare the differences between two samples and is often represented by a barplot. While relative abundance is an important part of the analytics for microbiome research significant pitfalls exist. Relative abundance does not take the total number of organisms into account, and thus when comparing across samples with differing bacterial load, the results can be misleading. For example, a stacked bar in a relative abundance barplot can represent a sample containing a total of 1,000 organisms while the other stacked bar has 10,0000 in total (Figure 2B); while the composition and percentages may be the same, the number of organisms can be significantly different.

Contamination

As mentioned earlier, contamination might occur during analysis. By sample retrieval, by kits used or by the way of handling by the researcher. The extent can be impactful and wrong conclusions can be drawn. Salter et al showed that bacterial contamination is characteristic to each laboratory, reagent, and reagent's lot. It is therefore recommended to include several negative controls in experiments. (46) When the sample has enough biomass the contamination is only a small proportion and can be eliminated with analysis. Bacteria that are commonly seen in analyses that can be (but not necessarily have to be) contaminants often have their natural habitat in water and low amounts of their DNA seems to remain present in buffers and kit materials, these are amongst others: *Acinetobacter, Curvibacter, Delftia, Legionella, Methylobacterium, Novosphingobium, Ralstonia, Sphingomonas, Undibacterium* and *Variovorax.* (47)

Diversity analysis

Taxonomic analysis allows the determination of the relative abundance of taxa within that bacterial community. To gain further insight into the bacterial composition of the samples usually a diversity analysis is performed. The two main techniques for diversity analysis are alpha diversity (α-diversity) and beta diversity (β-diversity). (48,49) Alpha diversity represents the within sample differences and is determined by the richness and evenness of members of the bacterial community within a single sample. Beta diversity represents the between sample differences and is the dissimilarity between communities of two sites (or two samples). Higher α-diversity means there are increased amounts of different taxa within on sample, whereas increased β-diversity means that composition between two communities are more different.

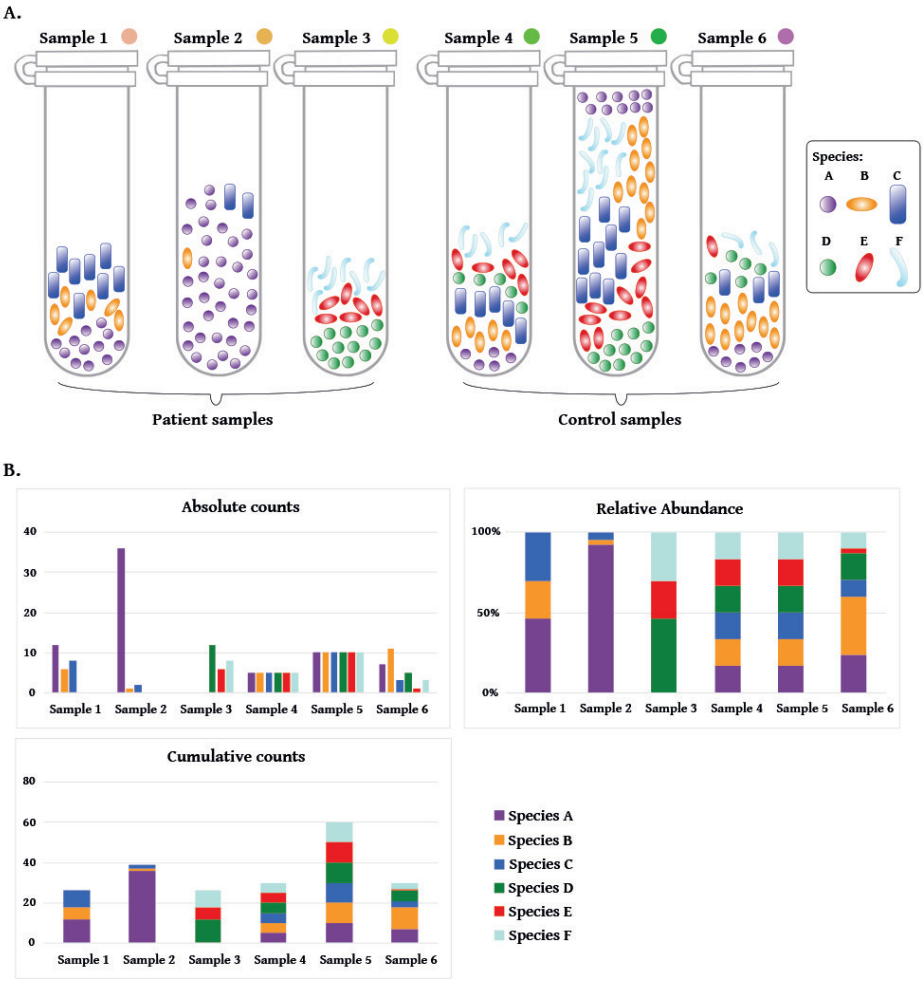


Figure 2 - A simplified example of 6 fictional samples with fictitious species – **A.** Three "patient" samples of a research group and 3 "control" samples, with various "species". Sample 1 (n=26) has the same species as Sample 2 (n=39), but in other amounts. Sample 3 (n=26) has different species than Samples 1 and 2, but has equal amounts of species as Sample 1. The control samples contain of all the species that are also present in the artificial samples. Sample 4 (n=30) and 5 (n=60) have the same proportions, but Sample 5 has twice the amount. Sample 6 (n=30) has the same species as the other controls but differs in ratio of the different species. **B.** Barplots are usually plotted as relative abundance plots. The relative abundance is sometimes interpreted wrongly, as it only presents the total composition. The barplots with absolute counts or cumulative counts are usually not presented, but are here plotted to show the difference with the relative abundance plots.

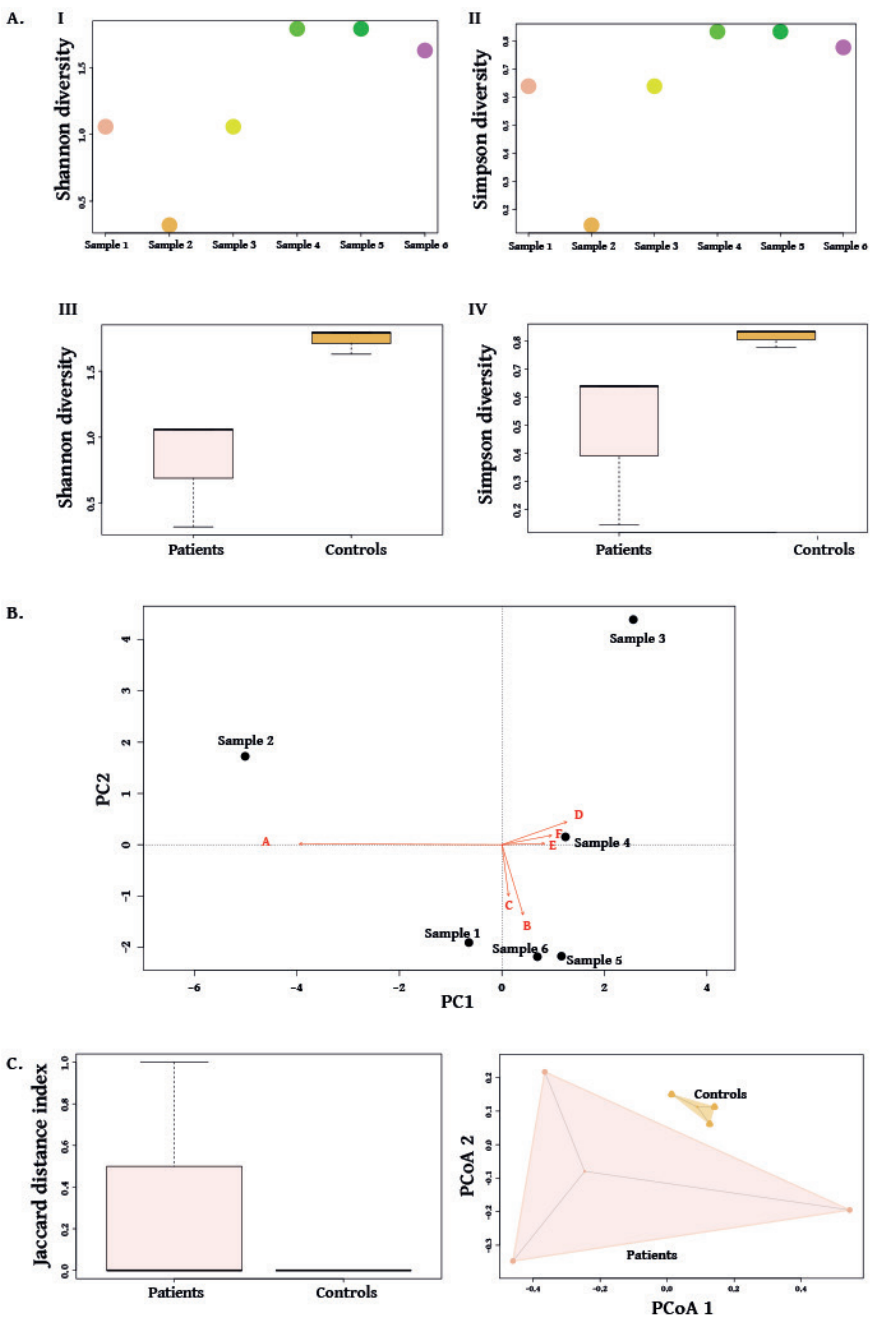


Figure 3 - Diversity analyses of example samples (shown in *Figure 2*) – A. Alpha diversity (**I**: Shannon and **II**: Simpson diversity) analyses per sample show that the samples with the most different species (sample 4 and 5) have a high diversity within the samples. The total amount of species does not matter, as long as the proportions are the same. Sample 6 is a little less diverse, due to different proportions. This is also the reason for the low diversity in Sample 2 compared to Sample 1. It has the same species but differs

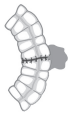
in proportion. Sample 3 is as diverse as Sample 1, but with different species. When grouped alpha diversity analyses (**III** and **IV**), the control samples show to have a statistically higher ($P < 0.05$) diversity with Shannon Diversity ($P = 0.02$, t-test) but not with the Simpson diversity index ($P = 0.11$, t-test). **C.** If we perform a Principal Component Analysis (PCA) and plot the first two principal components, the samples are plotted apart corresponding the direction (red arrow) of the different species. We see that Sample 2 lies far from the other samples, due to its relatively large amount of species A. Sample 3 is the only sample containing solely species 4, 5 and 6 and is therefore distant from other samples. Sample 4, 5 and 6 consist of the same species and are plotted close together, although differences in amounts of species separate them. Thus, the direction of the samples in this method are based on the presence and number of different organisms in the samples. This method is not commonly used, but does show the influence of the individual species (or the taxonomic rank of choice). **D.** In order to see the difference in diversity between the samples, beta diversity distance matrices can be used. When looking at the Jaccard Distance boxplot, there is a clear overlap between the two groups (artificial and control) and does not show statistically significant difference ($P = 0.37$, t-test). A Principal Coordination Analysis (PCoA) of the Bray Curtis dissimilarity shows that the samples within the group of artificial samples lie far apart, mainly due to the different abundances of the different species between the samples. However, they do not seem to overlap with the control samples. The diversity between the groups do not significantly differ ($P = 0.14$, t-test).

Numerous methods have been developed to create the most accurate representation of the diversity in or between samples. These methodical differences mainly differ by how they account for rare species. The Simpson's Diversity Index and Shannon Diversity Index are often used for α-diversity analysis. Shannon index assumes that all species are represented equally in a sample and that they are randomly sampled. In this case, a rare species with a small overall abundance will not affect the diversity. Alternatively, the Simpson's index is dependent on the dominance of species, and therefore when the dominance of a species increases, the diversity decreases (Figure 3A).

Similarly, β-diversity can be analysed in a number of different ways. A simple way of comparison diversity between samples is the Jaccard's index, representing the fraction of species shared between the samples without taking account the abundance of species. Bray Curtis dissimilarity works the same, but does consider the abundance of species (Figure 3B). Another widely used method for β-diversity is the UniFrac distance metric. It uses the phylogenetic distance between species in the samples, meaning that when species are more similar there is less diversity. UniFrac can be weighted (quantitative), where the abundance of species is taken into account or unweighted (qualitative) where abundance is not analysed by rather the presence of absence of the species in question.

Multiple testing

16S rRNA gene sequencing gives millions of reads for each sample analysed, depending on the sequence and sample size. Due to this huge amount of data, analyses should be corrected for multiple testing. Commonly used methods are Benjamini-Hochberg (also False Discovery Rate, FDR) or Bonferroni (Family-Wise Error Rate, FWER), with the first



the most used for bacterial research. Bonferroni uses all p-values and corrects them independently of their rank. This can create false negatives in large data, causing less significant observations. An FDR adjusted P-value (or q-value) can be interpreted like "normal" P-values, an FDR of 0.05 implies that 5% of significant tests result in false positives. Consensus about the cut-off value for FDR hasn't been reached and values <0.25 are sometimes considered to be significant, usually an FDR of 0.05 is used.

From mice or man?

Naturally, the bacterial composition differs between mice (*Mus musculus*) and human (*Homo sapiens*), but it is very important for the evaluation of microbiome-based manuscripts to take in mind where the samples studied were obtained from. The choice for a mouse model is tempting, but 85% of the sequences in mice that represent genera have not been detected in humans. (50) Besides, the bacterial richness (i.e. the number of different genera in the population) in mice seems to be higher. However, on phylum level the bacterial communities in both human and mouse seems to be similar, where the two main bacterial phyla are Bacteroidetes and Firmicutes. (50,51) Mice have large amounts of the phylum Deferribacteres, where only the human stomach hosts small amounts of. (52) There seems to be a higher abundance of *Prevotella, Faecalibacterium* and *Ruminococcus* in human samples, whereas in mice *Lactobacillus, Alistipes* and *Turicibacter* are more abundant. Genera like *Clostridium, Bacteroides* and *Blautia* are present in similar amounts. However, this data does differ per strain of mice and other confounding factors like mouse house origin and environmental conditions (food composition, light, stress factors, pathogen infection). (53) The use of 16S rRNA gene sequencing in mouse models should therefore be critically evaluated.

Other frequently used animal models are rat and pig models. Although having their practical disadvantages, their gut microbiota might be more similar to the human microbiota than mice. The gut bacterial communities of especially humanized rats are more similar to the gut microbiota of human. (54) The pig gut microbiota is interesting due to the similarities in anatomy, physiology and immunology to the human gastrointestinal tract. On a phylum level pigs have the same ratio of Bacteroides and Firmicutes as humans, but have more of the genera *Spirochaetes* and *Prevotella* than the human gut microbiota. (55) These differences make translation from research in other mammals to the human situation difficult.

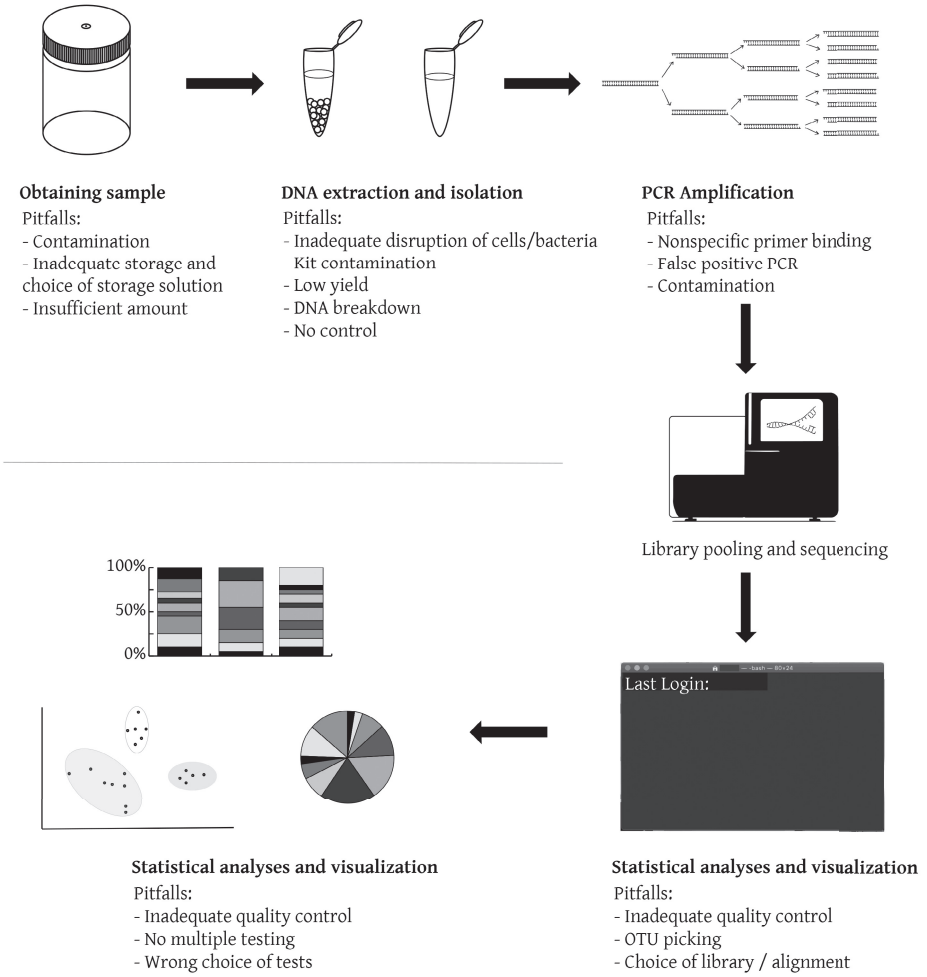
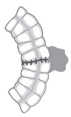


Figure 4 – Overview of the different consecutive steps (clockwise) in microbiome research and their pitfalls

Metagenomics and metatranscriptomics

So, the function of the bacteria and their interaction with the hosts organs solely analysed with 16S rRNA gene sequencing data remains a guess and can only be based of functions described on strain level research. For that shotgun metagenomic sequencing is needed. Metagenomic sequencing gives the opportunity to sequence all the DNA in a sample, thus sequencing thousands of genes from hundreds of microorganisms in parallel. This allows to create an approximation of the metabolism of individual microbial species and gain insight into their potential role in the interaction with the host. Metagenomic sequencing is conducted via shotgun sequencing, where the entire genome is broken up into small fragments of DNA and subsequently sequenced and analysed. For this all sequence reads can be mapped against available genome



reference databases or they can be reassembled using overlapping nucleotides in the attempt to recreate genomic species of the microbes present. It's technically challenging and time-consuming. Currently it is still a relatively expensive method, although sequencing prices still tend to go down. Performing these studies might pay off eventually, by facilitating the understanding of the mechanisms behind the disease of the investigated subject when the taxonomic and metabolic diversity of a sample are analysed.

In addition to this, RNA sequencing can be performed. This shows the gene expression (or transcriptome) of all genes from an organism present. One step further is metatranscriptomics. This is RNA sequencing of all RNA material present in the sample (from host and microorganisms). With that data the actual interaction between organisms can be analysed, or the influence of interventions can be observed. However, this method does not include the identification of the organisms the genes are from.

18S and ITS analysis

16S rRNA gene analysis is the golden standard for identifying bacterial (prokaryote) communities. However, in order to sequence eukaryotes or fungi respectively 18S rRNA and inter transcriptional spacer (ITS) sequencing between 18S rRNA and 28S rRNA are used. The method is in principle the same: amplicon sequencing, but the analysis needs more specialistic knowledge, which we will not elaborate in detail. Fungi account for <0.1% of the total microorganisms in the gut but can cause disease, especially when due to antibiotics or other invasive factors the microbial composition is disturbed. (56) It seems that fungi (and maybe even some eukaryotes) can have unexpected influence on results (Chapter 7 of this thesis).

DISCUSSION

The trend of rising interest in microbial research brings confusion about the used methods, analyses and the interpretation of results of these papers. Therefore, we have addressed some questions that arise on the topic of intestinal microbiome research, mainly focusing on 16S rRNA gene sequencing. Although the potential of this microbial identification method has shown to be very valuable, the method is unfortunately not perfect (yet). It's dependent on many variables, from DNA-isolation to data analysis, and is subject to interpretation. It therefore needs to be performed with precision and some caution.

For the surgeon this field is relatively unexplored. Even when microbiome research was performed from a surgical perspective, it was until now mainly limited to the intestinal microbiota as the subject of research. The gut, however, isn't the only

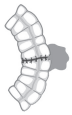
organ that can be studied for microbial presence and composition. Recently, it has been shown that (dormant) bacteria even occur in body fluids formerly seen as sterile, such as blood and urine. (57,58) The bacteria present in blood already have been linked to chronic diseases such as atherosclerosis, cardiovascular disease and diabetes type II. (59-61) It has been postulated that they might even hide in leukocytes, using them as "trojan horses" awaiting a signal to cause infection. (62) And amazingly, although the blood/brain barrier prevents the brain from bacterial invasion, the brain might even have its own microbiota. Human brain tissue collected at autopsy from pathologically normal brains were compared to abnormal brains from patients with HIV/AIDS and all showed to have bacterial populations. The patients' microbiota in the brain did differ to the normal brain, probably due to a permeable blood/brain-barrier. (63) The field has become broad and there's a gap to bridge in surgical research.

In this review we've tried to explain in a comprehensive way how to perform microbial research and what the possibilities are of culture-independent microbial analyses. We are only beginning to unravel the most complex symbioses there is: between mammals and microorganisms. Hosts influence their microbial inhabitants by behaviour, food and other intake, that directly has implications for the host's own health due to the microorganisms response. (64) It's implications for the surgical world could reach further than we might think.

An absolute advantage is that 16S rRNA gene sequencing allows a culture-independent analysis. The behaviour of microorganisms in their natural habitat is differs profoundly from their behaviour in culture. In culture there's a lack of interactions between other bacteria and host, as well as a difference in nutrients and resources. It doesn't give information on molecular level about the interaction between the host and microorganism but gives insight in the bacterial changes by treatments of the host. Besides, cultures show only culturable bacteria and do not necessarily represent the total composition. Ideally, for optimal research on the interaction between cells and bacteria, cellular models mimicking host's cell behaviour including the presence of bacteria are needed.

We can't conclude much with 16S rRNA gene analysis at the moment, except for changes in composition, in combination with the interpretation of known mechanisms of bacterial genera. Therefore, compared to metagenomics and (meta)transcriptomics it is limited, but it is better defined in the manners of usage. Besides, it's cheap and accessible and is a perfect method to give direction to future mechanistic research. If any functional data is needed besides the 16S rRNA gene sequencing, but (meta) transcriptomics or metagenomics are not feasible, reverse transcriptase PCR can be performed. If that has to be species specific, a good old culture might still be as valuable as it's ever been.

CONCLUSION



Novel sequencing methods are becoming an important analysis method for microbial diversity and should be used in surgical research. Current methods are not perfect yet to analyse or interpret microbial data. There is no universal protocol, there are very diverse methods, that can be interpreted differently. However, when one takes the subjects discussed in this review in consideration, conducting 16S rRNA gene sequencing can be a standard method for microbial research that surgeons should consider.

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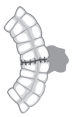

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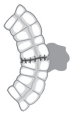

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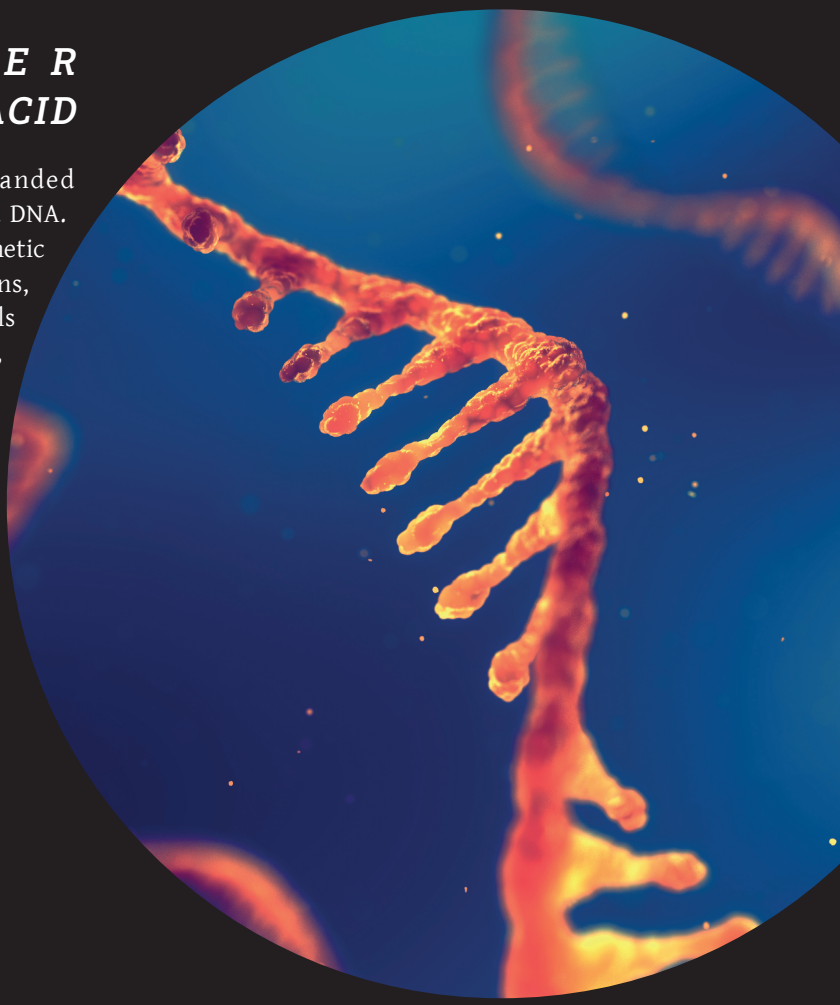
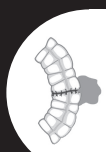
MESSENGER RIBONUCLEIC ACID

mRNA is the single-stranded transcript of double-stranded DNA. It functions to convert the genetic information of DNA to proteins, the building blocks of all cells in the human body. Therefore, mRNA is the fundamental component of gene expression and can be used to evaluate the (short term) effects of interventions (e.g. drugs).





CHAPTER 5

TRANSCRIPTOMIC PROFILES OF SAMPLES FROM COLORECTAL ANASTOMOSIS OF PATIENTS DEVELOPING ANASTOMOTIC LEAKAGE SHOW A DISTINCT SIGNATURE

Submitted

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ABSTRACT

Objective

To gain insights in the biological processes and pathways underlying the development of anastomotic leakage (AL) in patients undergoing colorectal surgery.

Design

We utilized a set of human samples from the anastomotic site collected during the stapled colorectal anastomosis. We generated RNAseq profiles of both patients developing AL and patients with normal healing of the anastomosis to identify genes and biological pathways associated with the development of AL.

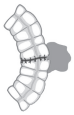
Results

Differential expression analysis shows that after quality control 44 genes have an adjusted P-value <0.05 consisting of 2 upregulated and 42 downregulated genes. Co-functionality analysis by the GenetICA framework of the top 150 most upregulated and most downregulated genes show formation of clusters of genes that show different enrichment for biological processes. The enriched processes for the downregulated genes are involved in immune response, angiogenesis, protein metabolism and collagen crosslinking. The enriched pathways for upregulated genes are involved in collagen metabolism.

Conclusion

Our data indicate that patients who eventually develop AL start the healing process with an arrear at the level of gene regulation at the moment of surgery AL. Despite normal macroscopic appearance during surgery, our RNAseq data identifies several differences in gene expression between patients who develop AL and patients who do not. The involved genes and the enriched processes are involved in the different stages of wound healing. These findings can be used to find therapeutic or diagnostic targets for the colon at risk for the development of AL prior to the creation of the anastomosis.

INTRODUCTION



Colorectal resection with restoration of continuity is a commonly performed surgical procedure. A frequent complication in ~10% of patients with a (primary) colorectal anastomosis is anastomotic leakage (AL), which is associated with prolonged hospital stay, reintervention, intensive care admission, permanent ostomies and even death. (1-3) The value of commonly used preventive measures, like deviating ostomies, omentoplasties and bowel preparation are often discussed in literature, but lack convincing evidence and AL still occur. (4-9)

Many factors are associated with an increased risk for the development of AL; poor perfusion of the bowel, increased tension on the anastomosis, technically imperfect anastomosis, chronic use of immune suppressive agents and comorbidity like diabetes or atherosclerosis. (10,11) In addition, it has been shown that AL is associated with a low diversity of the mucosal microbiome and presence of specific bacteria such as *E. faecalis* at the site of the primary colorectal anastomosis. (12,13) Multiple factors associated with mucosal injury and healing have been studied in their interaction with microbiota at the site of the anastomosis. (14) Some authors have associated intestinal healing with immune related factors such as cytokines and Toll-Like receptors (TLRs). (15-19) However, much remains unknown about the biological signalling pathways involved in normal or impaired colonic wound healing and thus the pathophysiology of AL.

Therefore, we aimed to gain insights into the biological processes and pathways underlying the development of AL. We utilized a unique set of human samples from the anastomotic site; the 'doughnuts' collected during the stapled colorectal anastomosis. Patients' characteristics and the postoperative course, including development of AL, were collected within the context of a prospective randomized trial. (20) Of these doughnuts we generated RNAseq profiles to identify genes and biological pathways associated with the development of AL.

METHODS

Sample acquisition

The samples used for this study were obtained from patients who participated in the C-seal trial. (20) The C-seal trial evaluated the effect and efficacy of the C-seal, an intraluminal device designed for the prevention of clinical anastomotic leakage in stapled colorectal anastomosis. In this multicentre trial 41 hospitals participated in the Netherlands, Germany, France, Hungary and Spain. The primary endpoint was AL requiring reintervention. The trial was open for inclusion from December 2011 until January 2014. The study was approved by the Medical Ethics Committee of the

University Medical Center Groningen and all participating centres. The trial was registered in The Netherlands National Trial Register under the number NTR3080.

For patients who gave additional consent, the doughnuts were retrieved and stored. These doughnuts are the small rings of colon and/or rectum that are cut by the circular stapler when the anastomosis is made. These samples, a proximal and distal sample, were removed from the stapler, directly put in RNA*later*® Solution (ThermoFisher Scientific, Waltham, MA) and subsequently stored in -80°C. For this study, the proximal samples were used. Twenty-nine patients who developed AL were matched on gender, age and preoperative chemotherapy and radiotherapy with ninety-four patients without AL. The samples of these patients were used for this study.

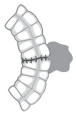
RNA isolation

Total RNA was extracted from proximal doughnuts using a protocol involving a combination of bead beating and the Maxwell® 16 LEV simplyRNA Tissue Kit (Promega, Madison, WI). Briefly, up to 60mg of tissue sample, 245µl of 1-Thioglycerol/ Homogenization Solution (provided with the Kit) and minibead glass beads were added to an Eppendorf tube and disrupted in a bead beater for 3x 45 seconds. Subsequently the homogenates were heated at 70°C for 2 minutes and cooled for approximately 1 minute. Samples were then centrifuged at 13,200×*g* for 5 minutes, and 200µl of supernatant transferred into new tubes with 200µl of Kit Lysis Buffer. Then the samples were vortexed and transferred into Maxwell® 16 LEV Cartridge Purification Kit cartridges. An additional 10µl of Kit DNAse I was added to the other wells of the cartridges described by the protocol, and 40µl of nuclease-free water was used for elution. The following RNA extraction protocol was carried out by the Maxwell® 16 Instrument according to the manufacturer's instructions (Promega).

RNA quality check

A quantity and quality (purity and integrity) check was assessed by measuring absorbance at different wavelengths using a NanoDrop spectrophotometer (Thermoscientific). Only the RNA samples with a 260/280 ratio between 1.9 and 2.1 and 260/230 ratio greater than 2.0 were used.

An additional quality check of the extracted total RNA and total RNA quantification of the samples was done by capillary electrophoresis using the LabChip GX (Perkin Elmer). Samples were included for further analysis if; 1) the rRNA (28S/18S) Height Ratio was >0.8 and 2) the mean RNA quality score (RQS), which correlates with the RNA Integrity Number (RIN),(21) calculated by the LabChip was >5.0.



mRNA sequencing

cDNA fragment libraries were generated using the BiooScientific Nextflex mRNA sample preparation kits (Bioo Scientific Corporation) using the Sciclone NGS Liquid Handler (Perkin Elmer). In case of contamination of adapter-duplexes an extra purification of the libraries was performed with the automated agarose gel separation system LabChip XT (PerkinElmer). The obtained cDNA fragment libraries of the samples were sequenced by the Illumina NextSeq500 System (Illumina) using a single-end read with a read length of 75 base pairs in 4 pools of 24 samples.

Quality control of sequencing data

Quality control (QC) was done per sequenced read and per sample, the QC metrics were calculated with the raw sequencing data (FastQ files) by using the tool FastQC (FastQC/0.11.3-Java-1.7.0_80). Subsequently all the reads where aligned to the reference genome *build 37 human* ensemble Release 75 using HISAT (hisat/0.1.5-beta-goolf-1.7.20) with default mode (searching for one or more alignments, each will be reported), allowing for 2 mismatches in the alignment. (22)

Raw sequencing metrics for the aligned reads were calculated and used to create a summary table using the Picard tools (picard/1.130-Java-1.7.0_80) CollectAlignmentSummaryMetrics and CollectRnaSeqMetrics, and by using SAMtools (SAMtools/1.2-goolf-1.7.20).(23) SAMtools (command *view-h)* was used to sort the aligned reads by name. Following, a gene level quantification in raw counts was performed by HTSeq Count (HTSeq/0.6.1p1) using default settings and the commands --mode=union --stranded=no and, Ensembl version 75 was used as gene annotation database. (24)

Differential gene expression analysis

Differential expression of the genes was analysed with DEseq2 package in R version 3.4.3. We first prefiltered (filtering out genes that do not have at least 3 samples with normalized counts greater than 10) and releveled the conditions as described by the authors. (25) Hereafter, the differential analysis was done (command *DEseq*), which gives a list of up- and downregulated genes based on the log2FoldChange and p-value. (25) In addition, p-values were adjusted for multiple comparisons by Benjamini-Hochberg False Discovery Rate (alpha = 0.05) (P_{adj}). Results were considered significant when $P_{adj}<0.05$.

Heatmap and hierarchical clustering

A heatmap was created with the 150 most upregulated and 150 most downregulated genes based on adjusted P-value of our data set using the online available ClustVis web

tool. (26) The clustering distances are based on Pearson correlation subtracted from 1. The linkage method used is the Ward linkage method.

Co-functionality network analysis

We utilized a guilt-by-association (GBA) approach that predicts likely functions for genes based on gene co-regulation (manuscript in preparation). In short, a covariance matrix was calculated between 19,635 genes using the expression patterns of 106,462 gene expression profiles generated with Affymetrix HG-U133 Plus 2.0. Consensus-ICA was performed on the covariance matrix, which resulted in the identification of a large set of independent components (ICs) and a mixing matrix (MM) reflecting the activity of each IC in the expression pattern of each gene across the samples. Next, a GBA approach was used to predict the functionality of individual genes. First, 16 public gene set collections were collected describing a large range of biological processes and phenotypes. For each gene set, a 'bar code' was calculated by averaging the MM weights of its member genes per IC. Next, for each gene in the MM, the distance correlation was determined between its MM weights and the gene set bar code. A high correlation between a gene's MM weight and a gene set bar code indicated that the gene under investigation shared a functionality with the genes of the specific gene set under investigation. Significance levels were obtained with permutated data (250 permutations). This strategy was used on 23,372 well-described functional gene sets, which enabled researchers to create a comprehensive network of predicted functionalities of individual genes. This framework is available at http://www.genetica-network.com.

Based on this framework the likelihood for every gene set as defined in a selected data base can be calculated. This will result in a vector of *n* likelihoods for each individual gene. The number of gene sets in the selected database determines the number *n*. Next, the correlation between the vectors of likelihoods of the different genes is calculated. The GenetICA framework then shows a network of genes in which all genes have at least a correlation with another gene above with a least one correlation above the selected threshold. When selecting a clustered network of genes, it shows the enriched function of the GO BP gene sets and its enrichment value (Z-transformed p-values), considered significant when Z-score>1.96. For our data set we used the 150 most upregulated and 150 most downregulated genes based on adjusted P-value. We used a threshold of 0.600 to separate clusters of genes. Clusters with at least 10 genes were used to adequately perform the enrichment studies.

RESULTS

Patient characteristics

The analysis was started with 123 doughnut samples, 29 of these were from patients who developed AL. After quality control of extracted RNA and sequencing results, we were able to continue our analysis with 91 samples. Out of these 91 samples, 22 samples were from patients that developed AL within 30 days after surgery, except for one patient (AL after 40 days), and 69 from patients that did not develop AL. Baseline characteristics for both patient groups used for subsequent analyses are presented in Table 1. With the exception of BMI (P=0.02) no significant differences were observed.

Table 1 *Patient Characteristics. Comorbidities are defined as disease under specialists' treatment. Mann-Whitney U test is used unless otherwise stated. § Welch's t-test, # Pearson chi-square Test.*

	Total (n = 91)	**Anastomotic leakage (n = 22)**	**No anastomotic leakage (n = 69)**	***P-value***
Age (years) (*sd*)	64.0 (*10.3*)	65.0 (*10.5*)	63.7 (*10.3*)	*0.63§*
Gender (Male/Female)	64 / 27	17 / 5	47 / 22	*0.44*
BMI (kg/m²) median *(25th – 75th quartile)*	26.5 *(24.0 – 29.4)*	27.7 *(24.8 - 31.2)*	25.9 *(23.8 - 28.2)*	*0.02*
Indication for surgery				*0.16*
Colorectal cancer	85	22 *(100%)*	63 *(91%)*	
Diverticular disease	4	0 *(0%)*	4 *(6%)*	
Other	2	0 *(0%)*	2 *(3%)* *	
Comorbidities				
Cardiovascular	42	11 *(50%)*	31 *(45%)*	*0.68*
Pulmonic	14	5 *(23%)*	9 *(13%)*	*0.29*
Neurological	11	3 *(14%)*	8 *(12%)*	*0.80*
Gastrointestinal	21	6 *(27%)*	15 *(22%)*	*0.59*
Urogenital	16	6 *(27%)*	10 *(14%)*	*0.17*
Musculoskeletal	15	5 *(23%)*	10 *(14%)*	*0.37*
Endocrine	16	3 *(14%)*	13 *(19%)*	*0.93*
Infectious	1	0 *(0%)*	1 *(1%)*	*0.57*
Concomitant malignancy (curatively treated)	17 [15]	4 [4] *(18%)*	13 [11] *(19%)*	*0.95*
ASA-score (ASA 1/ 2/ 3)	18 / 61 / 12	7 / 12/ 3	11 /49 / 9	*0.26*
Chronic corticosteroid	1	0 *(0%)*	1 *(1%)*	*0.57*
Preoperative treatment				
Chemoradiotherapy *50 Gray [Only chemotherapy]*	19 [1]	4 [0] *(18%)*	15 [1] *(22%)*	*0.72*
Short course radiotherapy - *25 Gray [with chemotherapy]*	31 [2]	7 [0] *(32%)*	24 [2] *(35%)*	*0.80*
Deviating ileostomy	0	0 *(0%)*	0 *(0%)*	*1.00*
Deviating colostomy [Hartmann]	5 [1]	1 [0] *(5%)*	4 [1] *(6%)*	*0.82*
During surgery				
New ostomy	40	8 *(36%)*	32 *(46%)*	*0.41*
C-seal	47	13 *(59%)*	34 *(49%)*	*0.42*

**Other: Adenoma / recurrent pelvic leiomyosarcoma*

A subset of genes shows significant differential expression between AL and non-AL samples

Four hundred and sixteen genes were significantly (P-value <0.05) upregulated and 1479 downregulated in AL samples compared to non-AL samples. 44 of those genes had a P_{adj}<0.05 consisting of 2 upregulated and 42 downregulated genes (Figure 1). Table 2 shows all genes with P_{adj}<0.05. The differential expression of all genes of AL samples versus non-AL samples can be found in Supplementary File 1 (available in online version).

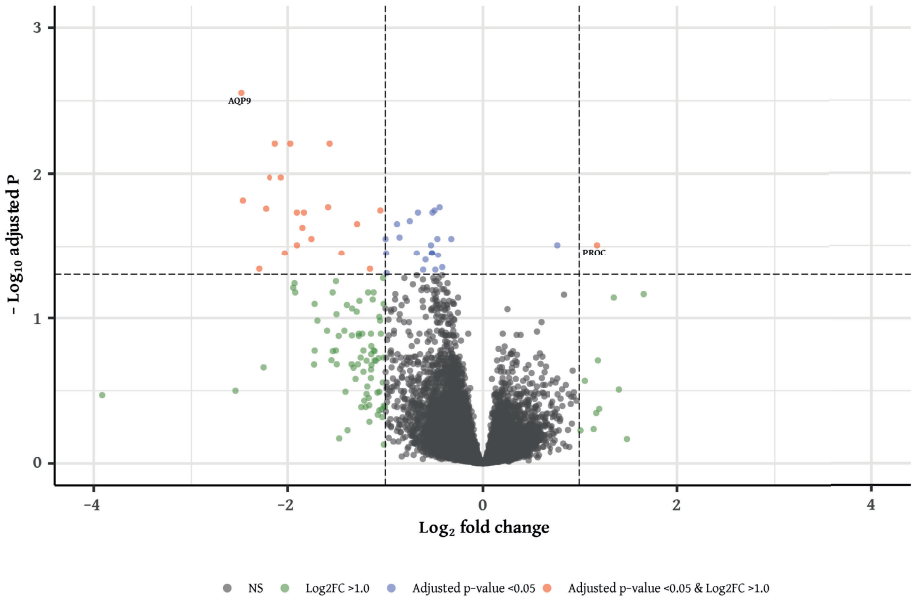


Figure 1 – A vulcano plot of all the genes, showing in grey the non-significant genes, in blue the genes with an P_{adj} < 0.05 and in green the genes with a Log2FoldChange >(-)1.0, and in red an P_{adj} < 0.05 and a Log2FoldChange >(-)1.0.

Table 2 – List of genes with an adjusted P-value (P_{adj}) less than 0.05.

Gene symbol	Gene name	Log2FoldChange	P-value	Adjusted P-value
PROC	Protein C, inactivator of coagulation factors Va and VIIIa	1.182	4.35E-05	3.10E-02
RP4-740C4.7	RP4-740C4.7	0.776	4.48E-05	3.10E-02
AQP9	Aquaporin 9	-2.489	1.41E-07	2.79E-03
HCAR3	Hydroxycarboxylic acid receptor 3	-2.473	5.38E-06	1.53E-02
MMP10	Matrix metallopeptidase 10	-2.305	9.41E-05	4.58E-02
PROK2	Prokineticin 2	-2.235	8.74E-06	1.74E-02
FCAR	Fc fragment of IgA receptor	-2.193	3.21E-06	1.06E-02

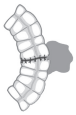


Table 2 (Continued) – List of genes with an adjusted P-value (P_{adj}) less than 0.05.

Gene symbol	Gene name	Log2FoldChange	P-value	Adjusted P-value
FCGR3B	Fc fragment of IgG receptor IIIb	-2.132	1.11E-06	6.22E-03
APOBEC3A	Apolipoprotein B mRNA editing enzyme catalytic subunit 3A	-2.070	2.91E-06	1.06E-02
LUCAT1	Lung cancer associated transcript 1	-2.030	6.35E-05	3.54E-02
CXCR2	C-X-C motif chemokine receptor 2	-1.973	1.15E-06	6.22E-03
CXCR1	C-X-C motif chemokine receptor 1	-1.905	1.35E-05	1.85E-02
MTRNR2L8	MT-RNR2 like 8	-1.905	4.52E-05	3.10E-02
FPR2	Formyl peptide receptor 2	-1.849	2.37E-05	2.35E-02
KCNJ15	Potassium voltage-gated channel subfamily J member 15	-1.833	1.40E-05	1.85E-02
MMP3	Matrix metallopeptidase 3	-1.757	3.53E-05	2.81E-02
SLC11A1	Solute carrier family 11 member 1	-1.586	7.23E-06	1.70E-02
CSF3R	Colony stimulating factor 3 receptor	-1.570	1.25E-06	6.22E-03
FPR1	Formyl peptide receptor 1	-1.449	6.00E-05	3.54E-02
MGAM	Maltase-glucoamylase	-1.289	2.02E-05	2.22E-02
SELL	Selectin L	-1.157	9.45E-05	4.58E-02
SLC16A7	Solute carrier family 16 member 7	-1.051	1.08E-05	1.79E-02
EMP1	Epithelial membrane protein 1	-0.997	3.33E-05	2.81E-02
NAMPT	Nicotinamide phosphoribosyltransferase	-0.991	6.40E-05	3.54E-02
NAMPTL	Nicotinamide phosphoribosyltransferase-like	-0.986	1.09E-04	4.91E-02
RP11-443N24.1	RP11-443N24.1	-0.880	2.12E-05	2.22E-02
HGF	Hepatocyte growth factor	-0.855	2.90E-05	2.74E-02
ZBED6	Zinc finger BED-type containing 6	-0.749	1.81E-05	2.12E-02
SLC2A13	Solute carrier family 2 member 13	-0.678	6.07E-05	3.54E-02
IKZF2	IKAROS family zinc finger 2	-0.666	1.49E-05	1.85E-02
IL6ST	Interleukin 6 signal transducer	-0.612	9.88E-05	4.65E-02
N4BP2	NEDD4 binding protein 2	-0.588	7.55E-05	3.95E-02
JMJD1C	Jumonji domain containing 1C	-0.532	4.44E-05	3.10E-02
CHD1	Chromodomain helicase DNA binding protein 1	-0.524	6.20E-05	3.54E-02
GPR126	G protein-coupled receptor 126	-0.522	6.24E-05	3.54E-02
AHR	Aryl hydrocarbon receptor	-0.518	5.87E-05	3.54E-02
AGO2	Argonaute 2, RISC catalytic component	-0.517	1.27E-05	1.85E-02
DOCK4	Dedicator of cytokinesis 4	-0.495	1.03E-05	1.79E-02
QSER1	Glutamine and serine rich 1	-0.488	1.01E-04	4.65E-02
SOCS4	Suppressor of cytokine signaling 4	-0.467	3.53E-05	2.81E-02
B4GALT6	Beta-1,4-galactosyltransferase 6	-0.459	6.81E-05	3.66E-02
DENND4A	DENN domain containing 4A	-0.444	7.69E-06	1.70E-02
ZNF800	Zinc finger protein 800	-0.417	8.76E-05	4.47E-02
PCGF5	Polycomb group ring finger 5	-0.325	3.21E-05	2.81E-02

Heatmap with hierarchical clustering shows three outlying samples

Figure 2 shows a heatmap of the top 150 most significantly upregulated and top 150 most significantly downregulated genes in patients who developed AL. Most (15 of 22) of the AL samples showed a similar pattern in their gene expression, since they clustered together. The differences in up- and downregulation in the samples of patients who develop AL (1) and who do not (0) were seemingly dichotomous and were homogenous within the groups of samples, with the exception of 3 samples. These 3 samples (#42, #118 and #123) belonged to the non-AL group and showed a different, outlying pattern compared to other non-AL samples. They had a higher upregulation in a subset of genes (see Figure 2, *MMP3* to *TLR4*).

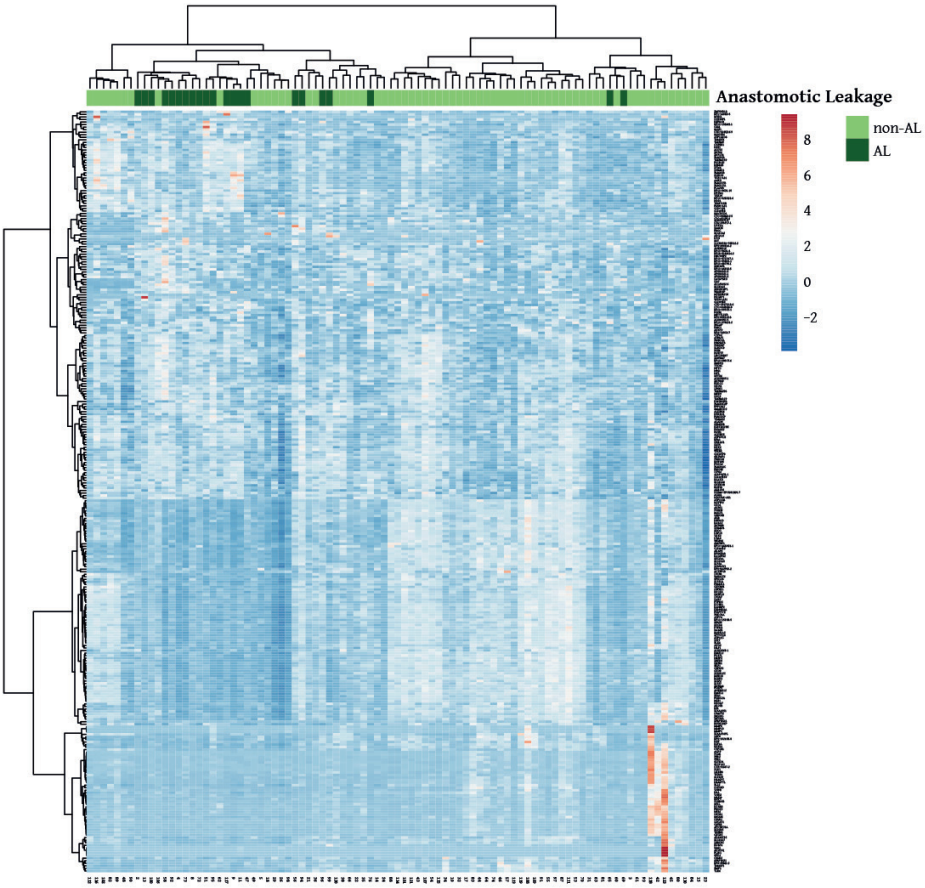


Figure 2 – Heatmap of differentially expressed genes with hierarchical clustering. The top 150 downregulated and top 150 upregulated genes are shown (rows) for each analysed sample (columns). The two side colour bars encode for (left) variance-stabilizing transformation of the count data and labels for (right) the groups non-AL (light green) and AL (dark green).

Co-functionality network identifies clusters of genes with pathways that have different signatures

The GenetICA co-functionality network of the top 150 upregulated genes consisted of 8 clusters of genes, ranging from 2 to 11 genes sharing predicted co-functionality. Only cluster 1 contained more than 10 genes (Figure 3), showing enrichment of the pathways GO organelle fission (Z-score 5.60), GO tRNA metabolic process (Z-score 5.36), GO DNA repair (Z-score 5.31), GO amide biosynthetic process (Z-score 5.17) and GO ncRNA Processing (Z-score 5.02). Because all these pathways were related to cell division, we labelled this cluster 1 '*cell division*'. See Table 3 for all upregulated pathways for cluster 1.

The clusters formed by the downregulated genes consist of 2 to 38 genes (Figure 4). Three clusters contained more than 10 genes. Cluster 1 showed 37 genes that were enriched for pathways in the *immune response*, such as GO leukocyte activation (Z-score 7.77), GO defence response to bacterium (Z-score 6.98), and GO defence response to other organism (Z-score 6.94). Cluster 2 (16 genes) enriched for pathways that involve *angiogenesis*: GO vasculature development (Z-score 4.72), GO blood vessel morphogenesis (Z-score 4.34) and GO angiogenesis (Z-score 4.33). Cluster 3 (24 genes) enriched for pathways such as GO mRNA processing (Z-score 5.43), GO DNA repair (Z-score 5.33) and the pathway GO peptidyl lysine modification (Z-score 5.05). The pathways of cluster 3 were related to *protein synthesis and collagen crosslinking*. See Table 4 for all downregulated pathways per cluster.

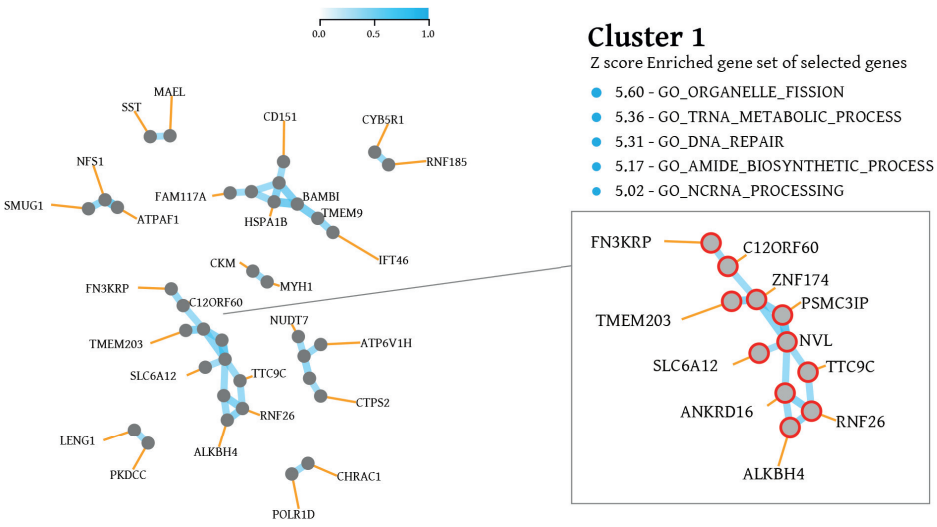


Figure 3 – This figure shows the co-functionality network of the top 150 upregulated genes within the GO Biological Processes. The threshold in the GenetICA framework was set at 0.600. The network consists of 8 different gene clusters sharing co-functionality, with the largest cluster containing 11 genes (cluster 1).

Figure 4 – The co-functionality network of the top 150 down regulated genes with the GO Biological Processes (threshold of 0.600). These genes aggregate in 3 larger clusters. Clockwise the first and largest cluster (cluster 1) contains 37 genes, the second cluster (cluster 2) consists of 16 genes and cluster 3 consists of 24 genes.

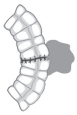


Table 3 - List of the pathways enriched by the top 150 up regulated genes in the cluster consisting of more than 10 genes (as displayed in Figure 3) with their Z-scores.

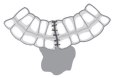
Cluster 1
5.60 - GO_ORGANELLE_FISSION
5.36 - GO_TRNA_METABOLIC_PROCESS
5.31 - GO_DNA_REPAIR
5.17 - GO_AMIDE_BIOSYNTHETIC_PROCESS
5.02 - GO_NCRNA_PROCESSING
4.89 - GO_REGULATION_OF_MITOTIC_CELL_
4.71 - GO_RIBOSOME_BIOGENESIS
4.50 - GO_CELL_DIVISION
4.38 - GO_RIBONUCLEOPROTEIN_COMPLEX_B
4.38 - GO_MITOTIC_NUCLEAR_DIVISION
4.24 - GO_MACROMOLECULAR_COMPLEX_DISA
4.22 - GO_RRNA_METABOLIC_PROCESS
4.20 - GO_CHROMOSOME_SEGREGATION
4.15 - GO_SPLICEOSOMAL_SNRNP_ASSEMBLY
4.14 - GO_TRNA_PROCESSING
4.14 - GO_NUCLEAR_CHROMOSOME_SEGREGAT
4.09 - GO_RNA_CAPPING
4.02 - GO_SISTER_CHROMATID_SEGREGATIO
3.99 - GO_DNA_REPLICATION
3.97 - GO_REGULATION_OF_CELL_CYCLE_PH
3.96 - GO_CELLULAR_PROTEIN_COMPLEX_DI
3.92 - GO_REGULATION_OF_DNA_METABOLIC
3.89 - GO_CELL_CYCLE_PHASE_TRANSITION
3.86 - GO_MRNA_PROCESSING
3.85 - GO_NEGATIVE_REGULATION_OF_CELL
3.82 - GO_POSITIVE_REGULATION_OF_RESP
3.82 - GO_NCRNA_TRANSCRIPTION
3.81 - GO_PROTEASOMAL_PROTEIN_CATABOL
3.79 - GO_REGULATION_OF_SIGNAL_TRANSD
3.79 - GO_CELL_CYCLE_CHECKPOINT
3.78 - GO_TRANSCRIPTION_COUPLED_NUCLE
3.77 - GO_RRNA_METHYLATION
3.74 - GO_RNA_MODIFICATION
3.73 - GO_NEGATIVE_REGULATION_OF_PROT
3.73 - GO_DNA_DAMAGE_RESPONSE_DETECTI

Table 4 – List of the pathways enriched by the top 150 down regulated genes in the clusters consisting of more than 10 genes (as discussed in Figure 4) with their Z-scores.

Cluster 1	Cluster 2	Cluster 3
7.77 - GO_LEUKOCYTE_ACTIVATION	4.72 - GO_VASCULATURE_DEVELOPMENT	5.43 - GO_MRNA_PROCESSING
7.03 - GO_INFLAMMATORY_RESPONSE	4.51 - GO_RESPONSE_TO_GROWTH_FACTOR	5.37 - GO_RNA_SPLICING
6.98 - GO_DEFENSE_RESPONSE_TO_BACTERI	4.37 - GO_REGULATION_OF_EPITHELIAL_CE	5.33 - GO_DNA_REPAIR
6.96 - GO_REGULATION_OF_CELL_ACTIVATI	4.34 - GO_BLOOD_VESSEL_MORPHOGENESIS	5.05 - GO_PEPTIDYL_LYSINE_MODIFICATIO
6.94 - GO_DEFENSE_RESPONSE_TO_OTHER_O	4.33 - GO_ANGIOGENESIS	4.95 - GO_RIBONUCLEOPROTEIN_COMPLEX_B
6.90 - GO_ACTIVATION_OF_IMMUNE_RESPON	4.22 - GO_GROWTH	4.90 - GO_NUCLEAR_TRANSPORT
6.54 - GO_MYELOID_LEUKOCYTE_ACTIVATIO	4.16 - GO_EPITHELIAL_CELL_DIFFERENTIA	4.88 - GO_POSTTRANSCRIPTIONAL_REGULAT
6.53 - GO_LEUKOCYTE_CELL_CELL_ADHESIO	3.94 - GO_EXTRACELLULAR_STRUCTURE_ORG	4.77 - GO_NUCLEOBASE_CONTAINING_COMPO
6.43 - GO_CELLULAR_DEFENSE_RESPONSE	3.92 - GO_MORPHOGENESIS_OF_AN_EPITHEL	4.72 - GO_REGULATION_OF_CHROMOSOME_OR
6.32 - GO_RESPONSE_TO_BACTERIUM	3.91 - GO_SKELETAL_SYSTEM_DEVELOPMENT	4.67 - GO_COVALENT_CHROMATIN_MODIFICA
6.32 - GO_LYMPHOCYTE_ACTIVATION	3.89 - GO_RESPONSE_TO_STEROID_HORMONE	4.56 - GO_REGULATION_OF_DNA_METABOLIC
6.27 - GO_REGULATION_OF_LEUKOCYTE_MED	3.88 - GO_HEART_DEVELOPMENT	4.53 - GO_GENE_SILENCING_BY_RNA
6.22 - GO_LEUKOCYTE_MIGRATION	3.83 - GO_CELL_SUBSTRATE_ADHESION	4.49 - GO_RNA_SPLICING_VIA_TRANSESTER
6.20 - GO_IMMUNE_EFFECTOR_PROCESS	3.80 - GO_MORPHOGENESIS_OF_A_BRANCHIN	4.48 - GO_REGULATION_OF_MITOTIC_CELL_
6.15 - GO_POSITIVE_REGULATION_OF_CYTO	3.79 - GO_OSSIFICATION	4.44 - GO_ORGANELLE_FISSION
6.09 - GO_REGULATION_OF_INNATE_IMMUNE	3.76 - GO_REGULATION_OF_TRANSMEMBRANE	4.33 - GO_MACROMOLECULE_METHYLATION
6.08 - GO_GRANULOCYTE_MIGRATION	3.75 - GO_CONNECTIVE_TISSUE_DEVELOPME	4.32 - GO_RNA_LOCALIZATION
6.05 - GO_POSITIVE_REGULATION_OF_INNA	3.73 - GO_FORMATION_OF_PRIMARY_GERM_L	4.27 - GO_DNA_TEMPLATED_TRANSCRIPTION
5.97 - GO_CELL_ACTIVATION_INVOLVED_IN	3.71 - GO_POSITIVE_REGULATION_OF_EPIT	4.21 - GO_NCRNA_PROCESSING
5.96 - GO_POSITIVE_REGULATION_OF_DEFE	3.70 - GO_GASTRULATION	4.19 - GO_REGULATION_OF_SYSTEM_PROCES
5.93 - GO_REGULATION_OF_HOMOTYPIC_CEL	3.65 - GO_EPITHELIAL_CELL_DEVELOPMENT	4.19 - GO_GENE_SILENCING
5.89 - GO_LEUKOCYTE_DIFFERENTIATION	3.62 - GO_BRANCHING_MORPHOGENESIS_OF_	4.17 - GO_REGULATION_OF_GENE_EXPRESSI
5.78 - GO_REGULATION_OF_INTERLEUKIN_6	3.59 - GO_REGULATION_OF_CELL_SUBSTRAT	4.15 - GO_METHYLATION
5.77 - GO_CYTOKINE_PRODUCTION	3.59 - GO_OVULATION_CYCLE	4.11 - GO_CIRCULATORY_SYSTEM_PROCESS
5.76 - GO_DETECTION_OF_OTHER_ORGANISM	3.58 - GO_RESPONSE_TO_ACID_CHEMICAL	4.09 - GO_PROTEIN_SUMOYLATION

Table 4 – List of the pathways enriched by the top 150 down regulated genes in the clusters consisting of more than 10 genes (as discussed in Figure 4) with their Z-scores.

Cluster 1	Cluster 2	Cluster 3
5.73 - GO_ADAPTIVE_IMMUNE_RESPONSE	3.57 - GO_MUSCLE_STRUCTURE_DEVELOPMEN	4.09 - GO_MITOTIC_NUCLEAR_DIVISION
5.60 - GO_IMMUNE_RESPONSE_REGULATING_	3.54 - GO_REGULATION_OF_ENDOTHELIAL_C	4.08 - GO_DOUBLE_STRAND_BREAK_REPAIR
5.54 - GO_REGULATION_OF_IMMUNE_EFFECT	3.53 - GO_CELLULAR_RESPONSE_TO_ACID_C	4.04 - GO_NUCLEAR_EXPORT
5.54 - GO_POSITIVE_REGULATION_OF_CELL	3.53 - GO_CELL_JUNCTION_ORGANIZATION	4.02 - GO_RRNA_METABOLIC_PROCESS
5.52 - GO_LYMPHOCYTE_DIFFERENTIATION	3.51 - GO_SKIN_DEVELOPMENT	4.02 - GO_PROTEIN_DNA_COMPLEX_SUBUNIT
5.43 - GO_REGULATION_OF_HEMOPOIESIS	3.49 - GO_REGULATION_OF_MORPHOGENESIS	3.96 - GO_NEGATIVE_REGULATION_OF_CHRO
5.42 - GO_REGULATION_OF_LYMPHOCYTE_ME	3.48 - GO_UROGENITAL_SYSTEM_DEVELOPME	3.96 - GO_CELL_CYCLE_PHASE_TRANSITION
5.38 - GO_REGULATION_OF_LEUKOCYTE_PRO	3.46 - GO_REGULATION_OF_EPITHELIAL_CE	3.95 - GO_NUCLEUS_ORGANIZATION
5.36 - GO_CELL_CHEMOTAXIS	3.45 - GO_ENDOTHELIAL_CELL_MIGRATION	3.93 - GO_CELL_DIVISION
5.34 - GO_DISRUPTION_OF_CELLS_OF_OTHE	3.44 - GO_REPRODUCTIVE_SYSTEM_DEVELOP	3.91 - GO_MRNA_3_END_PROCESSING

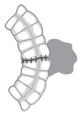


A subset of the downregulated genes in the cluster *immune response* were genes that were relatively downregulated due to the 3 previously mentioned outlying samples. This subset of genes accounted for a large part for the enrichment of GO defence response to bacterium, GO inflammatory response, and GO defence response to other organism. These pathways had lower Z-scores (≤5.00) when the subset genes were left out of the analysis. However, a cluster of genes that enriched for innate immune response related pathways like GO leukocyte activation (Z-score 9.52) and GO activation of immune response (Z-score 7.23), GO regulation of innate immune response (Z-score 6.90) were still present if these outliers were excluded from the enrichment analyses (See Supplementary Table 1).

DISCUSSION

This study presents a unique transcriptome analysis of 91 human colon samples from the anastomotic site and subsequent clinical outcome. Despite normal macroscopic appearance during surgery, our RNAseq data identifies several differences in gene expression between patients who develop AL and patients who do not. The majority of these genes are downregulated at the moment of surgery in patients who develop AL. These genes are individually interesting but should be put in the context of pathway regulation. A co-functionality network analysis shows 3 clusters of more than 10 downregulated genes in patients developing AL that can be labelled with the themes: *immune response* (cluster 1), *angiogenesis* (cluster 2) and *protein synthesis and collagen crosslinking* (cluster 3). The upregulated genes show one cluster with more than 10 genes, that seems to be involved in the *cell division* processes. This study provides the first analysis on transcriptomic level between patients that develop AL and patients that do not. We show a multifactorial transcriptomic signature of samples obtained right before the process of wound healing starts. These findings can be used to find targets for the colon at risk for the development of AL prior to the creation of the anastomosis. The identified enriched pathways can be used for the development of risk assessment and therapeutic strategies.

Our data show only two upregulated genes with an adjusted P-value <0.05. These genes are the PROC gene and RP4-740C4.7 (Table 2). The PROC gene regulates the creation of protein C, which is an important regulator of coagulation. Protein C is also considered to have an important anti-inflammatory effect via the Protein C pathway and is downregulated in active inflammatory bowel diseases. (27,28) The RP4-740C4.7 gene is located in the lead variant of Region 10 (rs6670198, Chr 1) of the human genome. Here it might affect the gene regulation of TNFRSF14 (a member of the TNF receptor superfamily), which encodes for a protein involved in signal transduction pathways



that activate both inflammatory and inhibitory T-cell immune responses. (29) The co-functionality network analysis showed one cluster of genes that seems to regulate for *cell division*. Pathways related to cell division, have shown to be upregulated in the connective tissue disorder Ehler Danlos. (30) In this disorder mainly collagen type V is negatively affected, which has a central role in fibrillogenesis and influences the activation of other collagen types. (31,32) Although the patients in the present study did not have Ehler Danlos, a negative influence on collagen type V could impair creation of extracellular matrix. However, uncontrolled upregulated cell division could also indicate presence of colorectal cancer cells, (33) but all samples had a macroscopic large tumour-free resection margin.

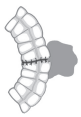
The identified downregulated gene clusters in the co-functionality network analysis can all be related to wound healing, in which three stages are identified: inflammation, proliferation and remodelling. There are no studies on the time-course of normal intestinal healing and most studies are focused on particular areas (such as epithelial cells) or diseases healing (such as IBD). (34,35) However, from a biological viewpoint it is likely that wound healing process within the human body largely works the same. Transcriptomic analysis of skin healing shows a distinct activation and upregulation of genes involved in the immune and inflammatory response, especially in the early stages of healing. (36) Wound healing studies in other tissue suggest the same upregulation in the defence mechanisms after injury. (37,38) Opposite to normal wound healing response, our results indicate that there is a downregulation of the innate immune response in patients developing AL compared to non-AL samples. Due to the presence of large quantities of bacteria, antimicrobial defence may be more important for wounds in the colon than for wounds in the skin. This is because the colon is more permeable as a result of the function to absorb and exchange between the lumen and the body compared to the skin, which is relatively impermeable. For instance because some bacteria have the virulent capabilities to cause degradation of the submucosal collagen at the anastomotic site. (13,39) Therefore, the downregulation of gene sets related to the immune response like GO leukocyte activation and GO activation of immune response (cluster *immune response*) in an environment with an overabundance of microorganisms could cause an unfavourable position for the healing colon after surgery and may be an important factor in the development of AL.

Previously we showed that in these samples a bacterial composition with low diversity seems to predispose for AL. (12) With stressors as surgery and administering antibiotics the commensal bacterial composition might be disrupted, which increases the chances for pathogens to thrive. In combination with an impaired immune response to bacteria, this could be detrimental for anastomotic wound healing. Additionally, there is an association between AL and some bacteria in particular,

such as *E. faecalis* and *P. aeruginosa.* (13,39) For example, *E. faecalis* is known to adhere to wound sites by upregulating its aggregation substance gene in response to stress situation, like surgery on the host or antibiotic treatment. Besides, *E. faecalis* activates its GelE gene which degrades collagen and activates MMP9, a degrader of extracellular matrix. Genes present in the downregulated cluster *immune response* like Aryl Hydrocarbon Receptor (AHR), aquaporin-9 (AQP9), suppressor of cytokine signalling 4 (SOCS4) and toll-like receptor 4 (TLR4) (see Table 1) are all genes involved in the innate immune processes. AHR is a crucial modulator of host-environment interactions and senses infection of amongst others the bacterium *P. aeruginosa.* (40) Activation of AHR signalling is important for the subsequent anti-bacterial response, with a critical role for macrophages and neutrophils modulation. (41) The AQP9 gene encodes for the aquaporin-9 protein which has bactericidal aspects, and is activated like AHR by bacteria such as *P. aeruginosa*. (42) Aquaporin-9 is also essential for cell migration during wound healing. (43) SOCS4 encodes for proteins that are key regulators of the innate and adaptive immune system as well. Additionally, TLR4 is a gene regulating the TLR4 protein that helps the immune system recognize microorganisms, in particular gram-negative bacteria and damage-associated molecular patterns (DAMPs) that are released during tissue damage caused by surgery. (44,45) Gram-negative pathogens like *P. aeruginosa* and *Serratia marcescens* have shown to be involved in the development of AL. (39)

However, the downregulated genes enriching for GO defence response to bacteria in AL-samples are mainly caused by high differential expression of 3 samples in the non-AL group. (Figure 2 and 4). Although these samples seem to be outliers, patient characteristics of these 3 samples are not divergent. It could be that these patients have such a high response to bacteria that they are unlikely to develop leakage caused by bacteria. Maybe there is a subset of patients that has this (relative) upregulation, but analysis of a larger cohort would be needed to conclude that. Another explanation could be that a (higher amount of) lymph node was included in the doughnut.

Our results also show the cluster *angiogenesis* with downregulated genes enriched for amongst others GO vasculature development and GO angiogenesis pathways. Angiogenesis, resulting in adequate supply of oxygen and nutrients, is one of the pillars of the proliferative phase of wound healing. (46) The healing of colonic anastomoses is considered to be more dependent on angiogenesis (microvasculature) than on diffusion of oxygen through pre-existing macrovasculature. (47,48) The use of anti-angiogenic agents, such as bevacizumab, has been associated with delayed wound healing and increased AL. (49,50) It has also been shown that impaired angiogenesis due to the lack of COX-2 gene in knockout mice causes a higher leak rate. (51) While application of vascular endothelial growth factor (VEGF) causes augmentation of angiogenesis and



enhances anastomotic healing. (52,53) The actual angiogenesis in intestinal healing usually starts after 3 days after the creation of a wound. (46,54)

The remodelling phase of the wound healing process is mainly collagen restructuring. Anastomotic healing, which can be considered as secondary wound healing, starts with granulation. As the clotting matrix is transformed a collagen network is formed, reducing the wound defect. Therefore, the balance of collagen production is an essential part of the healing of the anastomosis. (13,48,55) The fusion of the submucosal collagen matrix, which provides strength to the bowel, is essential for good intestinal wound healing. (46) Our data shows that one of the downregulated pathways is the GO Peptidyl Lysine Modification, which is involved in collagen crosslinking. (56) This may be another factor predisposing for anastomotic leakage. The genes involved in this pathway have a co-functionality with other pathways involved in protein metabolism (Figure 4).

A criticism of this study could be that the genetic impact of the colorectal cancer that most patients had was not taken into account. We do not think, however, that this was of any influence on our samples. As said, all samples had a free tumour resection margin. The top genes as shown in Table 1 are not known to be regulated by cancer or cancer treatment. Furthermore, radiotherapy causes a downregulation of extracellular matrix organization and blood vessel development. (57) There were no differences in radiotherapeutic treatment between groups (Table 1).

This study provides new directions in the search for mechanisms behind AL. It seems that the focus should be moved to the biological status of the patient and the threats a disrupted barrier faces in the form of microorganisms. Our data provides genes and biological routes that could be targeted for modulation or guided imaging. They could be used for the risk-profiling or even the prediction for the development of AL. In addition, prevention aimed at modulation of the luminal environment or manipulation of the gut microbiota might help to reduce the possible pathogenic effects of these inhabitants. The application of selective decontamination at the anastomotic site gives less (3.3% vs 7.6% in control) AL by eradicating a significant proportion of the bacteria present. (58) However, eradicating bacteria has its down sides since they are also important in the healing of (intestinal) wounds. (14) A better solution might be to prevent bacteria to become virulent. A promising example has been shown with phosphorylated PEG, which reduces the virulence of intraluminal bacteria. (59)

CONCLUSION

Although there is no literature on how the pathways behave as soon as the anastomotic wound healing is started, we hypothesize that patients who eventually develop AL start the healing process with an arrear. Our data show that they have a status or a trait at the level of gene regulation at the moment of surgery that predispose them for AL. This predisposition is mainly based on the immune response, angiogenesis, protein metabolism and collagen crosslinking and are seemingly all involved in the different stages of wound healing. These findings can be used to further elucidate the mechanism behind anastomotic leakage.

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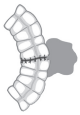

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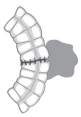

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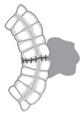
SUPPLEMENTARY FILES

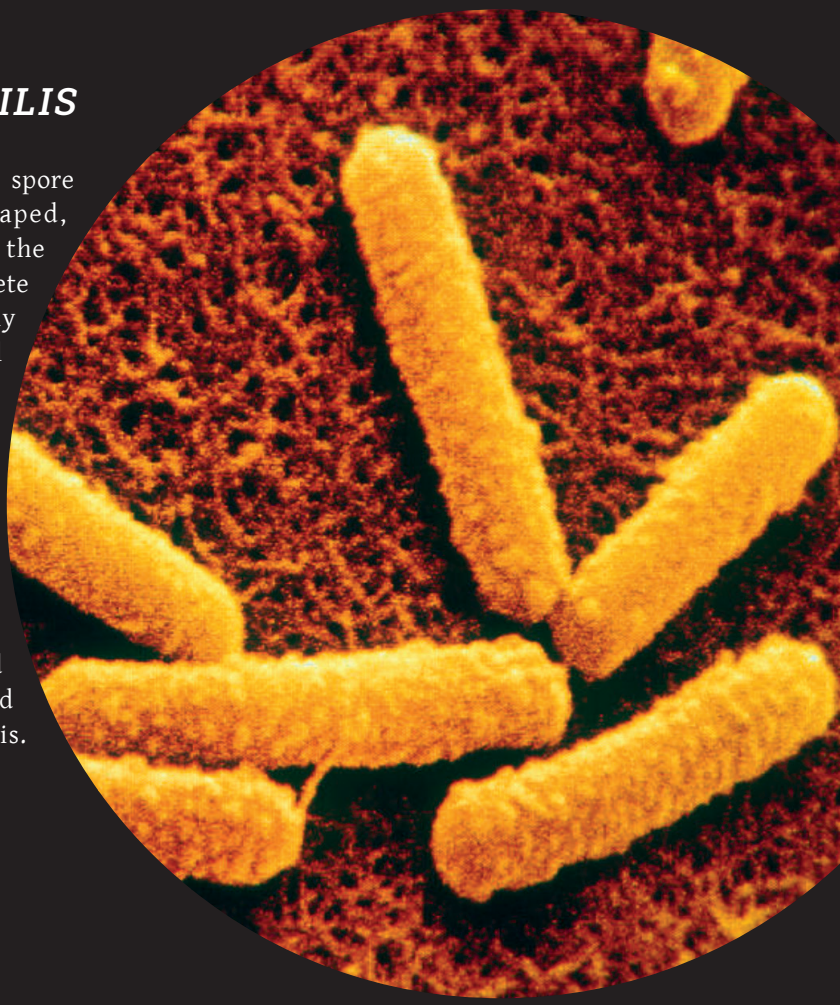
Supplementary Table 1 –This table shows the GO Biological Processes pathways that are enriched by the downregulated genes of cluster 1 without the subset of genes that are relatively downregulated due to outliers.

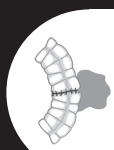
Cluster 1 – without outliers
9.52 - GO_LEUKOCYTE_ACTIVATION
8.85 - GO_LYMPHOCYTE_ACTIVATION
8.41 - GO_REGULATION_OF_CELL_ACTIVATI
8.13 - GO_LYMPHOCYTE_DIFFERENTIATION
8.09 - GO_LEUKOCYTE_DIFFERENTIATION
7.68 - GO_POSITIVE_REGULATION_OF_CELL
7.43 - GO_POSITIVE_REGULATION_OF_INNA
7.23 - GO_ACTIVATION_OF_IMMUNE_RESPON
7.20 - GO_REGULATION_OF_HOMOTYPIC_CEL
6.98 - GO_IMMUNE_RESPONSE_REGULATING_
6.90 - GO_REGULATION_OF_INNATE_IMMUNE
6.48 - GO_T_CELL_DIFFERENTIATION
6.47 - GO_REGULATION_OF_CELL_CELL_ADH
6.39 - GO_IMMUNE_EFFECTOR_PROCESS
6.36 - GO_REGULATION_OF_HEMOPOIESIS
5.95 - GO_POSITIVE_REGULATION_OF_DEFE
5.76 - GO_POSITIVE_REGULATION_OF_CELL
5.74 - GO_REGULATION_OF_LEUKOCYTE_MED
5.74 - GO_MYELOID_CELL_DIFFERENTIATIO
5.71 - GO_LEUKOCYTE_CELL_CELL_ADHESIO
5.66 - GO_REGULATION_OF_LEUKOCYTE_PRO
5.53 - GO_B_CELL_ACTIVATION
5.47 - GO_ADAPTIVE_IMMUNE_RESPONSE
5.36 - GO_LYMPHOCYTE_ACTIVATION_INVOL
5.33 - GO_CYTOPLASMIC_PATTERN_RECOGNI
5.25 - GO_REGULATION_OF_LEUKOCYTE_DIF
5.12 - GO_REGULATION_OF_ADAPTIVE_IMMU
5.11 - GO_T_CELL_RECEPTOR_SIGNALING_P
5.09 - GO_CELL_ACTIVATION_INVOLVED_IN
5.04 - GO_REGULATION_OF_ALPHA_BETA_T_
5.02 - GO_PRODUCTION_OF_MOLECULAR_MED
5.00 - GO_DEFENSE_RESPONSE_TO_OTHER_O
4.91 - GO_ANTIGEN_RECEPTOR_MEDIATED_S
4.88 - GO_REGULATION_OF_IMMUNE_EFFECT
4.83 - GO_PROTEIN_AUTOPHOSPHORYLATION



BACILLUS SUBTILIS

B. subtilis is a Gram-positive, spore forming, motile, rod-shaped, facultative aerobe. It has the ability to produce and secrete antibiotics. It is frequently found in the gastrointestinal tract of humans. It can form a tough, protective endospore, allowing it to tolerate extreme environmental conditions. To date, little is known about the virulence potential of this bacterium. This species is associated with anastomotic leakage and is amongst others discussed in Chapter 6 of this thesis.





CHAPTER 6

WORKFLOW AND METHODS TO DETERMINE THE ROLE OF BACTERIA IN ANASTOMOTIC LEAK PATHOGENESIS FROM HUMAN SAMPLES: DEFINING THE ROLE OF *BACILLUS SUBTILIS*

Submitted

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ABSTRACT

Background and purpose

Various bacterial strains have shown to contribute to anastomotic leak in gastrointestinal surgery. This study aims to define a methodology that links bacteria retrieved from an anastomotic leakage site to its potential role in leakage pathogenesis.

Methods

We obtained the anastomotic fluid sample from a patient following a total gastrectomy who developed an anastomotic leakage on postoperative day 3. We cultured and speciated a strain of *Bacillus subtilis* and determined its ability to express a "leak phenotype" as judged by its ability to produce collagenase, activate matric metalloprotease 9 (MMP9) and cause a clinical leakage when introduced to anastomotic tissues in a mouse model.

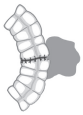
Results

The patient's culture was positive for *Bacillus subtilis* which produced a very high level of collagenase and activated MMP9. Introduction of the strain (n=7) into mice subjected to a colorectal anastomosis via enema fed both a standard diet (SD) and a high fat western type diet (WD) and exposed to antibiotics resulted in a statistically significant leak rate (SD- 14% leak rate, $p = 0.02$; WD- 50% leak rate $p < 0.01$ compared to controls).

Conclusions

Bacteria retrieved from anastomotic leakage sites may be identified to have the potential to contribute to anastomotic leakage pathogenesis when they are analysed beyond their species and antibiotic resistance profiles. Determining the "leakage phenotype" among strains retrieved from patients with anastomotic leakages may advance our understanding of the pathogenesis of this dreaded complication.

INTRODUCTION



Anastomotic leakage remains a real and present danger to patients despite improvement in operative technique, use of antibiotics and rapid availability of imaging. Although leakage rates across high volume centres seem to have significantly decreased over the last several decades, even among high volume expert surgeons, leaks still occur resulting in significant disability. Our group has been interested in bacteria as causative agents in anastomotic leakage across all sites of the gastrointestinal track. Yet, even with the use of standardized preoperative oral and intravenous antibiotics, certain commensal organism, with the potential to become pathogenic, survive antibiotic exposure and colonize anastomotic tissues. Our group has provided compelling evidence to uncover the multiple contingencies required for bacteria to behave as causative agents of anastomotic leakage. First the normal flora must be disrupted allowing certain strains of bacteria to proliferate such as those that can colonize injured tissues and produce toxic tissue degrading enzymes such as collagenase. Second, these bacteria must be "cued" by local environmental factors in the injured tissues such as the release of cytokines or end-products of ischemia/hypoxia. Third, these collagenolytic pathogens must activate tissue proteases that further impair collagen production and anastomotic healing such as matrix metalloprotease 9 (MMP9), which is known to play a key role in anastomotic leakage pathogenesis. (1) When all of these contingencies are met, certain bacteria can fulfil the criteria of expressing a "leak phenotype." Although it is common to culture various enteric organisms from an anastomotic wound site, discerning which of these are merely a reflection of the anastomotic wound itself versus actually contributing to the development of the wound has remained a challenge. We previously identified *Enterococcus faecalis* as an organism that fulfilled many of the criteria expressing the "leak phenotype." (1) Although *E. faecalis* is a low abundance commensal organism, it normally survives antibiotic exposure, can proliferate when the normal microbiota are eliminated and can shift its phenotype to become highly pathogenic. In this report, we describe a case of anastomotic leakage in which the retrieved microbe was found to be a commensal mouth organism normally not considered to be a pathogen. Yet when tested for its ability to fulfil the above criteria, it expressed the "leak phenotype" as judged by its ability to express collagenase, shift MMP9 from its pro-form to its active form and cause anastomotic leakage in rodents when introduced directly to the anastomotic tissue site.

Here we describe a workflow (Figure 1) in which the potential of a retrieved microbe from an anastomotic leakage site to express the leak phenotype can be determined. Yet whether a given microbe retrieved from an anastomotic wound site

plays a role in the pathogenesis of anastomotic leakage in humans, remains to be determined.

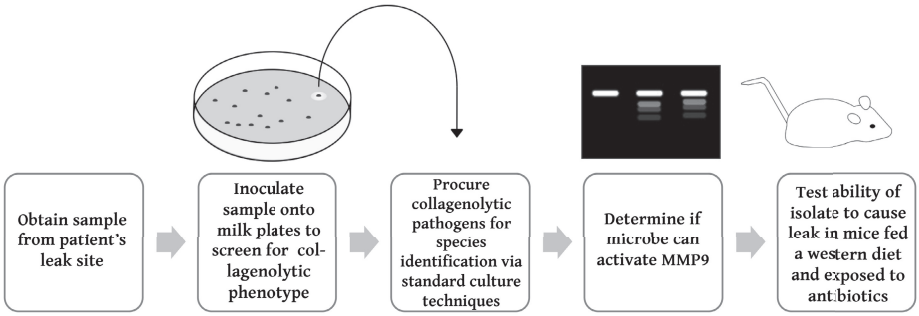
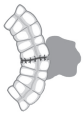


Figure 1 – Workflow to determine the possible contribution of microorganisms in a clinical anastomotic leakage.

METHODS

Patient history: A 48-year-old female patient, with an BMI of 35 and a known CDH-1 mutation had recently undergone bilateral mastectomies with reconstruction for breast cancer 7 months prior to the current presentation. In consultation with her medical oncologist, she elected to undergo prophylactic gastrectomy. (2) A laparoscopic total gastrectomy with a hand-sewn Roux-en-Y oesophagojejunostomy was performed in standard fashion. Intraoperative endoscopy at the conclusion of the procedure revealed an air-tight anastomotic with no evidence of ischemia. An UGI obtained on postoperative day 3 showed no evidence of leak the patient was given a clear liquid diet and advanced to pureed foods. The night of POD3 the patient spiked fever of 39.2°C and became mildly tachycardic. A CXR revealed free air, however the patient was in no acute distress, had no signs of peritonitis on physical exam, was normotensive and did not have any signs of sepsis. A decision was made to forego CT imaging and perform repeat laparoscopy. Approximately 50cc of turbid fluid was identified near the anastomosis and was taken for culture. Upper endoscopy was performed, and a 2 mm hole was observed without any grossly visible areas of ischemia. The upper abdomen was irrigated and drains were placed. The patient underwent placement of a stent which was eventually removed with complete healing of the anastomosis and without stricture. Follow up after several months of the index operation demonstrated that the patient was doing well, had no nausea and was eating regular meals. The patient, who was obese prior to the index operation, had lost 50 lbs (23kg), had a BMI of 27 and maintained a stable weight.



Identification of collagenolytic bacteria

The initial fluid sample obtained during the re-exploration demonstrated normal mouth and gut flora and was therefore the clinical laboratory did not perform additional cultures to identify the organism. Our laboratory retrieved the original fluid sample from the clinical lab and subjected it to a series of tests to determine if it expressed the "leak phenotype." Therefore, fluid samples were directly plated on skim milk-containing plates as recently described to determine if cultured organisms expressed collagenase. (3)

Briefly, plates specified for Gram(-), Gram(+), Enterococcus and Pseudomonas were created with either incorporation of skim milk into Enterococcus, MacConkey Gram(-), and Pseudomonas media, or by skim milk overlay of Columbia CNA Agar with 5% Sheep Blood (CNA, Gram(+)). (3) Colonies surrounded by zones of skim milk clearance (i.e. collagenase producing organisms) were collected and identified. Speciation of isolated collagenolytic colonies was then performed by the clinical microbiology laboratories at the University of Chicago Medicine and the Mayo Clinic Hospital in Rochester, Minnesota.

Quantitative collagenase assay

Quantitative analysis of isolated colonies for collagenolytic activity was performed using the EnzChek gelatinase/collagenase assay. Bacteria were grown overnight in TY media consisting of 10 g/L tryptone and 5 g/L yeast extract. They were then inoculated into 96 well plates at 1:100 dilution of overnight cultures with fresh TY media; fluorescein-conjugated gelatine from pig skin (D12054, ThermoFisher Scientific) at a final concentration of 5 µg/ml was introduced immediately before measuring using a multichannel pipette. Cell density (OD= 600 nm) and collagenolytic activity (495/515 nm, excitation/emission) were measured immediately (t=0) and serially for up to 24 hours. Start point values were subtracted, and collagenolytic activity was normalized to cell density.

MMP9 activation assay

Recombinant human proenzyme MMP9 (r-MMP9) (Calbiochem, catalog #PF038) was used as a substrate. The r-MMP9 was diluted to a final concentration of 1 µg/ml in the assay buffer [50 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$, and 0.05% Triton X-100]. 30 ml of *B. subtilis* grown in TSB (OD 600 nm = 1.0) was incubated with 20 ml of r-MMP9 (1 mg/ml) for 2 hours. After co-incubation, the conditioned medium was centrifuged (6,000g, 20 min), and 5 µl of the supernatant was mixed with an equal volume of 2× SDS loading buffers and subjected to zymography. Zymography for identification of zones of enzyme activity was performed using 7.5% SDS-PAGE gels containing 0.1%

gelatine as previously described. (4) *E. faecalis* strain E2, previously shown to activate MMP9, was used as a positive control.

Mouse model of anastomosis leakage in response to exogenous bacteria

In order to have easy access to an anastomosis in the mouse so as to be able to easily expose it to the test microorganism, we performed a colorectal anastomosis on mice as previously described. (5). Briefly, male BALB/c mice aged 6 weeks old (Charles River Laboratories) were used in the experiments. Mice were maintained in accordance with the University of Chicago IACUC Protocol 72491 with guidelines prepared by the University of Chicago Institutional Animal Care and Use Committee. Our protocol for this mouse model is to create conditions that mimic those of our patients with antibiotics exposure and feeding of a western diet. Therefore, mice are randomly assigned to two feeding regimens, a Standard low fat, high fibre diet (chow) diet (n = 14) and a high fat, low fibre Western diet (Bio Serve S3282) (n = 12) more typical of the diet of our patients. Mice were allowed their defined diet and water *ad libitum* for 6 weeks, and the weight of the mice was measured at the end of 6 weeks. Prior to the surgery, mice received oral clindamycin (100 mg/kg, ~50 mL oral gavage of 50 mg/mL) and a subcutaneous injection of cefoxitin (40 mg/kg, ~100 mL of 10 mg/ mL), mimicking the preoperative antibiotic treatment in human colorectal surgery. Subsequently the mice were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). In short, after opening the abdomen by a midline abdominal incision, the colon was transected at the peritoneal reflection and an anastomosis was created with 7 interrupted sutures of 8-0 proline. The anastomosis' integrity was verified by administration of an 100µl enema of normal saline via a 22-gauge blunt, olive tip needle, after which the abdomen was closed by a double layer of 5-0 Vicryl followed by a 5-0 Nylon. Animals were resuscitated with 1mL subcutaneous injection of 0.9% normal saline. Postoperative analgesia consisted of subcutaneous injections of buprenorphine (0.05mg/kg Henry Schein) every 8-12 hours for first 48 hours and one-time subcutaneous dose of meloxicam (1mg/kg Henry Schein). On postoperative day (POD) 1, 100 mL of a freshly prepared bacterial suspension with *B. subtilis* (OD600nm = 1.0 in 10% glycerol) or only vehicle (10% glycerol) was administered once via a rectal enema with a 22-gauge blunt olive tip needle. Half of each group was given the bacterial suspension (Standard diet n=7, Western diet n=6). The mice were observed during the postoperative days for clinical signs of leakage such as lethargy, weight loss, decreased movement and visible chills. Mice that exhibited signs of leakage were sacrificed and their anastomoses visually evaluated for signs of leakage. All other mice were sacrificed by CO_2 asphyxiation with confirmatory cervical dislocation on POD6, upon which their anastomoses were visually inspected at the time of sacrifice.

The anastomotic healing was scored with the validated anastomotic healing score (AHS) as previously described (5): 0 = normal healing; 1 = loose adhesion; 2 = dense adhesion; 3 = gross abscess formation; 4 = gross leak with peritoneal contamination.

RESULTS

Bacillus subtilis expresses the "leak phenotype" as judged by its ability to degrade collagen and activate MMP9.

The initial clinical microbiology culture results of the turbid fluid grew mixed normal mouth and gut flora and therefore was not speciated by the clinical microbiology laboratory. The fluid sample then was cultured on skim milk agar plates specified for Gram(-), Gram(+), Enterococcus and Pseudomonas. (3) Collagenolytic colonies were found on Gram(+) CNA plates covered with skim milk. The facultative anaerobe *Bacillus subtilis* was identified as a highly collagenolytic that predominated on the plates. Quantitative collagenase assay revealed high level of collagenase activity as seen by degradation of fluorescein-labelled gelatine. *B. subtilis*' collagenase activity was three-fold that of the other Gram(+) collagenolytic strains of *E. faecalis* isolated previously from anastomotic tissue in rat model of anastomotic leakage (Figure 2A). *B. subtilis* was also capable of MMP9 cleavage activation, similar to that of *E. faecalis* (Figure 2B).

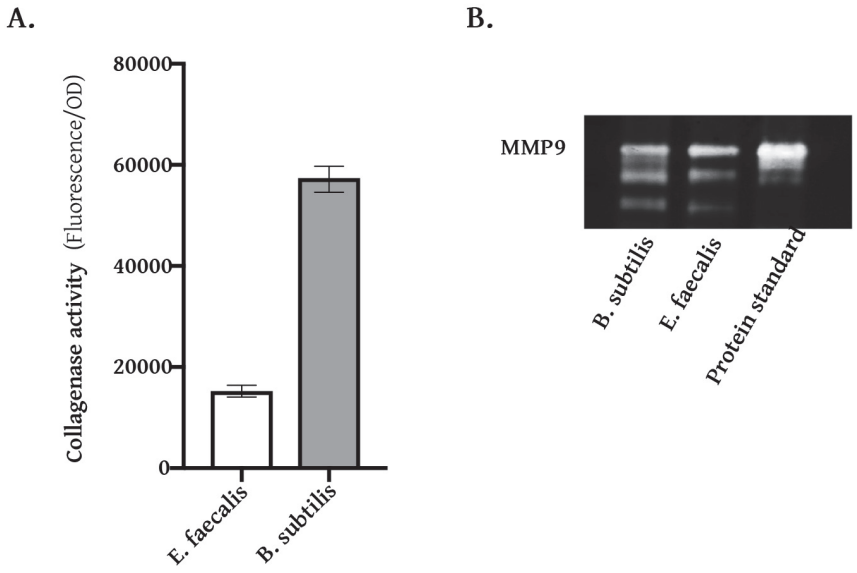


Figure 2 – Collagenolytic activity (A) Collagenolytic activity of the *Bacillus subtilis* strain compared with a known high collagenolytic *Enterococcus faecalis* strain (E2 (1)). **(B)** The *B. subtilis* shows to be capable of MMP9 cleavage activation similar to *E. faecalis*.

Exposure of *Bacillus subtilis* to the anastomotic tissues of mice results in impaired healing and gross clinical leakage.

In order to analyse the potential of *B. subtilis* to cause leakage, we tested the bacteria in our mouse model using mice on standard chow and a western diet. Mice fed Western diet for 6 weeks significantly increased in weight ($p < 0.01$, t-test) (Figure 3). In both the Standard diet and the Western diet groups, the mice given the saline enema (vehicle control) did not develop any visible signs of anastomotic leakage when sacrificed on postoperative day 6 (POD6). In the Standard diet group with *B. subtilis,* only one mouse showed signs of leakage at POD6 and showed abscess formation at the anastomotic site during inspection after sacrifice. Three mice in the Western diet group with *B. subtilis* developed severe clinical signs of leakage on POD5 and were therefore sacrificed. In all three cases, upon evaluation of the anastomoses, there was visible dehiscence with significant perianastomotic adhesion, abscess formation and spilled luminal contents. Statistical analysis showed a difference in leak rate between the *B. subtilis* mice and the vehicle mice in the Standard diet group (14%, $p = 0.02$, Mann-Whitney U). In the Western diet group, there was also a statistically higher leak rate in the mice given a *B. subtilis* enema compared those who received only vehicle suspension (50%, $p = 0.04$, Mann-Whitney U). AHS in Western diet fed mice with *B. subtilis* was significantly higher compared to Standard fed mice with a vehicle suspension ($p < 0.01$, Mann-Whitney U) (Figure 4).

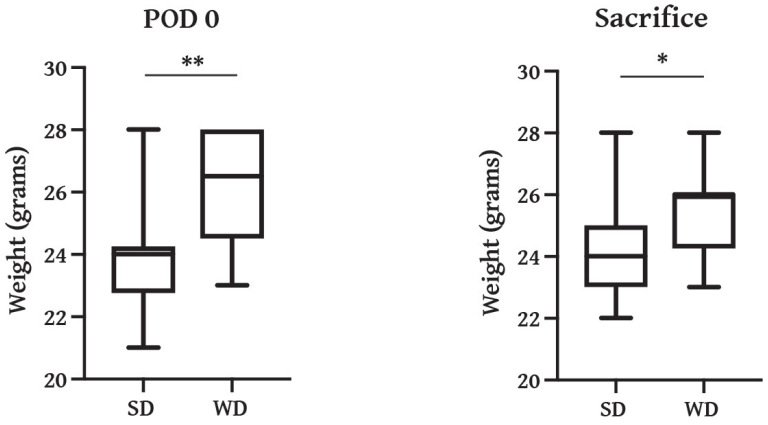
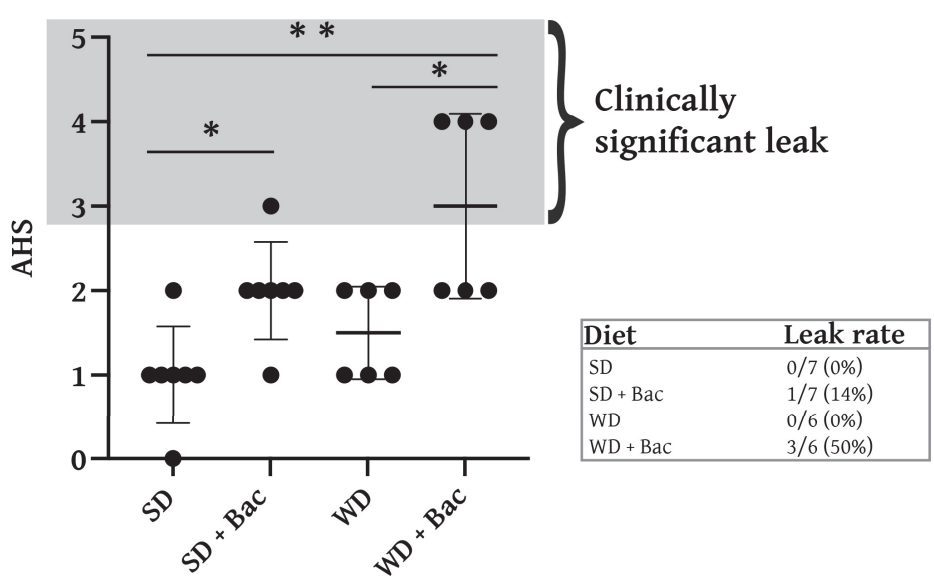


Figure 3 – Weight of mice (A+C) Weight of mice on the day of surgery (POD 0) **(B+D)** and on the day of sacrifice. The weight of the mice was significantly higher in mice fed a Western diet (WD) on POD 0 ($p < 0.01$, t-test) and on POD 6 ($p = 0.02$, t-test). On POD 0 the mice with a Standard diet (SD) and a *B. subtilis* enema differed in weight compared to mice fed a Standard diet with a vehicle enema ($p = 0.04$, t-test), but this difference had disappeared at the day of sacrifice. In addition, the mice fed a Western diet and given a *B. subtilis* enema had a significantly higher weight than mice fed a Standard diet given the same enema on both POD 0 and the day of sacrifice ($p < 0.01$).



Diet	Leak rate
SD	0/7 (0%)
SD + Bac	1/7 (14%)
WD	0/6 (0%)
WD + Bac	3/6 (50%)

Figure 4 – Anastomotic Healing Score (AHS) The anastomotic healing score of the 4 groups of mice. Mice fed a Standard diet (SD) given a *B. subtilis* enema have a significantly higher leak rate compared to mice with a vehicle enema ($p = 0.02$). Mice fed a Western diet (WD) group and given a *B. subtilis* enema leaked more compared to Western diet with vehicle enema and Standard diet with vehicle enema (respectively $p = 0.04$ and $p < 0.01$).

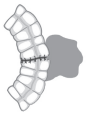
DISCUSSION

The results of this report and analysis provide a methodology to link a microbe retrieved from an anastomotic leakage site to its potential role in the pathogenesis of leakage. Although results of the present study suggest a potential role for *B. subtilis* in the leak observed in this case, the fact that the leak occurred on postoperative day 3 is highly suggestive of a technical issue such as tension, ischemia, suture placement, etc. Evidence against this possibility is the finding that endoscopy and UGI suggested integrity of the anastomosis following immediate construction and during 3 days of healing. However, the process of ischemia and non-healing could peak during the ensuing days following initial anastomotic construction and account for a non-bacterial mediated anastomotic leakage occurrence. Yet the extent to which colonization of a highly collagenolytic bacteria that can activate MMP9 on an anastomotic site with a technical issue contributes to the clinical manifestation of leakage, remains to be determined. (1,5) Developing a standardized methodology to understand the role of microbes in leak pathogenesis may require interrogation of the microbe beyond its speciation and antibiotic resistance profile alone. It is for this reason, mice were exposed to antibiotics and administered a high fat diet, both

of which are known to alter the microbiome and are a routine part of the care of a patient undergoing anastomotic surgery. (6)

The microorganism *Bacillus subtilis* is a human commensal organism that normally colonizes the gastrointestinal tract. While it is not traditionally considered to be a pathogen, it has been associated with foodborne illness. (7,8) It has been previously demonstrated to produce gelatinase and other *Bacillus* species are known to produce collagenase. (9) *B. subtilis* is highly resistant to extreme environmental conditions and antibiotics and in some cases it has been used as a probiotic to prevent infection. (10,11) However in some cases, as demonstrated in the present report, it may have the potential to behave as a pathogen. Like many commensal organisms, depending on environmental context, colonizing microbes can adapt a commensal lifestyle, while in other circumstances a pathogenic one. The current approach of intestinal antisepsis prior to gastrointestinal surgery does not consider microbial phenotype expression as a target of the antibiotics. Yet when competing organism are eliminated by broad spectrum antibiotics, remaining organisms can shift their phenotype, especially when they encounter environmental cues such as those present in a fresh intestinal wound. Therefore, a more complete understanding of the microbiome that patients bring to the operating room prior to surgery, the organism we eliminate with current antibiotic regimens and those that subsequently colonize healing tissues may allow for a more complete identification of organisms with the potential to complicate healing. Such information may indicate that a one-size-fits-all approach of antibiotic prophylaxis may not be ideal.

Data from the present study suggests that in the current era of promiscuous antibiotic use, the intake of a western type diet and the prevalence of obesity, identification of microbial organisms and the phenotype they express at sites of anastomotic leakage may be important to inform future approaches for intestinal antisepsis prior to gastrointestinal surgery. As microbiome sciences continue to develop, understanding which organisms to preserve and which to eliminate may be now technically possible in the era of advanced sequencing. Results from the present study demonstrate that both phenotype expression (i.e. collagenase production) and introduction of isolate into mice may play an important role in establishing the extent to which a microbe, present at the site of an anastomotic leakage, contributes to the molecular pathogenesis of this devastating surgical complication.



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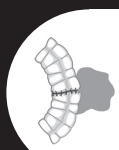
CANDIDA PARAPSILOSIS

C. parapsilosis is a fungal species of yeast. It is considered as a normal human commensal. However, patients with suppressed immunity are at risk for infection with this species. It is considered to be an emerging fungal pathogen and is associated with a high morbidity and mortality rate. This species is associated with anastomotic leakage and is amongst others discussed in Chapter 7 of this thesis.



CHAPTER 7

WESTERN DIET PROMOTES INTESTINAL COLONIZATION WITH COLLAGENOLYTIC MICROBIOTA AND DRIVES TUMOUR FORMATION FOLLOWING COLORECTAL SURGERY

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ABSTRACT

Background

A high-fat, Western diet is a modifiable risk factor for recurrence after curative resection of colorectal cancer.

Methods

Here we describe a novel murine model of tumour formation developing at the colon resection reconnection site (anastomosis) when mice consume a Western type diet and then anastomotic tissues are exposed to antibiotics, collagenase producing (collagenolytic) *Enterococcus faecalis* and dispersed mouse colon cancer cells.

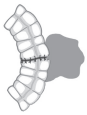
Results

This model produces a high incidence of extra-mucosal colon tumour formation that significantly correlates with the presence of collagenolytic *E. faecalis* and *Proteus mirabilis*. Use of additional antibiotics to eliminate collagenolytic *E. faecalis* and *P. mirabilis* fails to reduce tumor formation and promotes the emergence of an alternative, opportunistic collagenase-producing microorganism, *Candida parapsilosis*. Use of a previously characterized phosphate-carrying compound ABA-PEG20-Pi20 (Pi-PEG), known to suppress bacterial collagenase without affecting bacterial growth, reduces tumor formation and maintains microbial diversity.

Conclusions

These results have major clinical implications and may explain how cancer cells shed into the anastomotic environment during colorectal surgery can cause local recurrence and how factors such as diet and antibiotic use might be modified to prevent local-regional recurrence rates.

INTRODUCTION



Annually, nearly 140,000 new cases of colorectal cancer (CRC) are diagnosed in the US and despite advances in its treatment, CRC remains the 2nd and 3rd most common cause of deaths in men and women, respectively. (1) The lethal nature of CRC is attributed to a nearly 15% rate of local recurrence and 25% rate of distal recurrence after surgical resection with curative intent. (2) Although locoregional recurrence is presumed to be a function of microscopic tumour left behind during the index operation, emerging evidence suggests a more central role for the environmental biology of the primary tumour itself. (3) Unique to CRC is the inevitable presence of shed cancer cells in the lumen of the colon exfoliated from the primary tumour during growth or surgical manipulation. (4,5) While the surgical reconnection of the intestine following resection (termed an anastomosis) is intended to be impermeant, animal studies have determined that shed cancer cells can penetrate the anastomosis resulting in local recurrence. (6-8) However, the selective pressures present in the local anastomotic environment that promote shed cancer cells to penetrate an intact anastomosis and form tumours, remains incompletely understood.

Previous work from our laboratory has demonstrated that the anastomotic environment with its attendant tissue injury and systemic effects, can act as a chemoattractant for collagenase producing intestinal microbiota (i.e. *Enterococcus faecalis*) that can cause disruption of a surgically intact anastomosis. (9,10) Additional factors such as prior antibiotic use, preoperative chemo-radiation and diet may further promote these pathobionts to predominate in anastomotic tissues where they can cause tissue breakdown and clinical leakage. (11,12) Because anastomotic leaks are strongly associated with local CRC recurrence, it is possible that local conditions at the site of a disrupted anastomosis may create opportunities for shed cancer cells to penetrate tissue and form tumours. (13-16)

Multiple aspects of CRC including its incidence and pathogenesis are now known to be strongly influenced by the intestinal microbiome. (17-19) This is not surprising given that the intestinal microbiome mediates the interface between our environment and our genes. An example of this interface specific to the intestinal track are the dietary nutrients, which are emerging as having a profound effect on both microbiome composition and function. (20) The finding that patients consuming a high fat, Western type diet following curative colorectal cancer resection have higher rates of recurrence suggests a link between diet, its influence on the microbiome, and microbiome-mediated changes in intestinal epithelial function. (21,22) These linkages provide a proposed mechanism by which various disorders such as colon cancer, diabetes, obesity and autoimmune disease develop. (23,24)

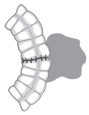
In the present study, we sought to combine various elements in the setting of colon cancer resections that might predispose to loco-regional tumour formation. These included the feeding of a high fat/low fibre Western diet, exposure to prophylactic antibiotics, introduction of strains of collagenolytic *Enterococcus faecalis*, and exposure of anastomotic tissues to dispersed (shed) colon cancer cells. We hypothesized that diet-induced alterations in the intestinal microbiota promote anastomotic penetration of dispersed colon cancer cells present in the lumen resulting in tumour formation. Therefore, the aim of the present study was to demonstrate that alterations in the intestinal microbiota that develop from the combined exposure to a Western diet, antibiotics and collagenolytic *E. faecalis*, can render anastomotic tissues susceptible to loco-regional tumour formation following luminal exposure to a murine colon cancer cell line. Results indicate that a technically adequate colorectal anastomosis can be rendered permeable to dispersed colon cancer cells when the microbiome is altered by a Western diet and antibiotics resulting in a predominance of a collagenolytic pathobiota in anastomotic sites.

MATERIALS AND METHODS

Mouse Model of Colorectal Anastomosis

BALB/c male mice aged 8-10 weeks (Charles River) were used in all experiments. The animals were maintained in accordance to protocol IACUC 72491 with guidelines prepared by the University of Chicago Institutional Animal Care and Use Committee. Mice were allowed food and water *ad libitum* throughout all experiments. By random assignment, mice were provided with standard Chow diet (std) or Western-type diet (WD) (Bio Serve S3282), characterized by high fat/low fibre/decreased minerals and vitamins composition as compared to Chow diet (Supplementary Table 1). Mice were maintained on their respective diets for four weeks prior to antibiotic exposure and weighed weekly.

We used a previously validated mouse model of distal colon anastomosis. (10) One hour prior to surgery, all animals received intramuscular cefoxitin, 40mg/kg (Henry Schein, Mellville, NY, USA) and per os clindamycin, 100mg/kg (Henry Schein, Mellville, NY, USA). All mice underwent general anaesthesia with intraperitoneal ketamine, 100mg/kg (Henry Schein, Mellville, NY, USA) and xylazine, 10mg/kg (Henry Schein, Mellville, NY, USA). In select experiments, animals were administered a rectal enema with a 22-gauge blunt olive tip needle with 100µl of control or experimental solution on post-operative day 1 or 2 as per study design. The enema was administered over one minute with mice placed in a tail-vein injector.



Anaesthetic complications occurred in less than 10% of animals. Additional animals were operated on and added to the study groups to achieve equal numbers. The remaining animals did well and appeared normal after surgery until the time of sacrifice on POD 21. Mice fed a WD were of the same weight as mice fed a chow diet which is consistent with previous studies demonstrating the resistance of BALB/c mice to weight gain when placed on a high fat diet. (25)

Bacterial Strain and Preparation for Administration

Enterococcus faecalis E2 used in this study was previously isolated from anastomotic rat tissue. (9) The E2 strain was grown on selective Enterococcal agar (BBL™ Enterococcosel™ agar; BD diagnostics, Sparks, Maryland, USA) for 24 hours prior to inoculation. Only cells freshly plated from stock frozen at -80°C in 10% glycerol were used in all experiments. On post-operative day 1, an E2 suspension was made with an optical density of 600nm = 0.5 in sterile 10% glycerol ($1x10^8$CFU/ml).

Murine Colorectal Cancer Strain and Preparation for Administration

Luciferase/tdTomato-labeled monoclonal (L2T) CT26 cells derivative of CT26.WT (ATCC CRL-2638) the colon carcinoma cell line derived from BALB/c mice were used in this study. The (L2T)CT26 were generated by the Ralph Weichselbaum lab at University of Chicago and provided as a gift. (26)

To create the aliquots used for injection, $1x10^6$ L2T CT26 (named as CT26 in the manuscript) was thawed and grown in Dulbecco's Modified Eagle Medium supplemented with 10% Fetal Bovine Serum, 100 U/mL penicillin, and 100 mg/mL streptomycin. CT26 was maintained in cell culture at 5% CO_2 and 37°C. Once cells reached 80% confluency, cells were removed from the plate with 0.05% trypsin, spun, washed, and resuspended in Hank's Balanced Salt Solution $1x10^5$ cells in 100µl were injected via enema as described above.

Animal Tissue Analysis and Collection

Animals were sacrificed on post-operative day 21 and the abdominal cavity was opened through the previous laparotomy scar. The peritoneum and liver were inspected for evidence of grossly visible metastases. The distal colon was resected proximally at the splenic flexure and distally at the rectal/anal margin in order to encompass the anastomosis and extra-luminal tumour. The colon was opened longitudinally and the luminal contents were removed. A portion of the faeces was placed in 500µl of sterile normal saline for subsequent DNA isolation. The remainder of the luminal contents was placed in sterile 10% glycerol for subsequent microbial culture. A portion of the distal colon and tumour (if present) were placed in 500µl of sterile normal saline for DNA

isolation and the remainder was placed in 10% glycerol for microbial culture analysis. In a subset of animals, the distal colon and tumour (if present) were submitted for histological analysis (see below). The determination of tumour was made by visual inspection and confirmed by histological analysis (see below) in selected samples. After these preliminary histological confirmations, the subsequent determination of subsequent tumour formation was made by visual inspection. For micrometastatic analysis via RT-PCR, the liver was removed in entirety and placed in 1.5mL of sterile normal saline (0.9% NaCl).

Histology and Trichrome Staining

Distal colon and tumour were submitted for histological analyses according to standard protocol (fixed in 10% formalin for 24 hours followed by washing in 70% Ethanol. Embedded tissue was cut in 5µm slices and subjected to H&E staining. Trichome staining was used to detect collagen.

Microbial Culture for Collagenase Activity

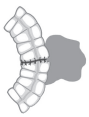
Homogenized colon tissue and luminal content aliquots were thawed and serially diluted. 20µl of the stock solution and each dilution was plated onto skim milk containing EnterococcoselTM agar (15% skim milk, US Biological Life Sciences, Salem, MA, USA), and MacConkey (Difco™ MaConkey, BD, Sparks, MD, USA) (20% skim milk) prepared as previously described. (10,27) Collagenolytic colonies were identified at in the Clinical Microbiology Laboratory at the University of Chicago. The CFU count was normalized to sample weight.

DNA Isolation

The collected mouse samples were homogenized with the FastPrep-24™ 5G Instrument (MP Biomedicals, Solon, OH, USA). Total DNA was extracted from 300 mL of homogenate with the FastDNA SPIN KIT (MP Biomedicals, Solon, OH, USA) according to manufacturer's protocol.

16S rRNA gene analysis

Total DNA isolate obtained from the mouse samples was used as template DNA. Each 25µl PCR reaction contained 12.5 µl of AccuStart II PCR ToughMix (Quantabio, MA, USA), 8.5 µl of PCR-grade water, 1 µl Forward Primer, 1 µl mPNA (50µM) (PNA BIO INC, CA, USA) to block host target mitochondrial contamination, 1 µl Golay Barcode Tagged Reverse Primer (5 µM), and 1 µl of template DNA. The PCR conditions were 94°C for 3 minutes followed by 35 cycles at 94°C for 45 s, 50°C for 60 s, and 72°C for 90 s with a final extension of 10 min at 72 °C. The resulting amplicons were subsequently



cleaned, pooled, and quantified using the Quant-iT picogreen double-stranded DNA assay kit following EMP benchmarked protocols (http://www.earthmicrobiome.org/emp-standard-protocols/). Pooled amplicons were then pair-end sequenced (2x 150bp) on Illumina MiSeq sequencing run at the Argonne National Laboratory, IL, USA.

Bacterial 16S V4 region (515F–806R primer pair) (28,29) sequences were processed using Qiime2 (v2018.6). (30) Default parameters were used for demultiplexing. Quality control was performed with the DADA2 function integrated in Qiime2 to truncate forward and reverse reads, to denoise the data and for detection and removal of chimeras. (31) The representative sequences variants of each sample were retained and assigned to bacterial taxa using a Naive Bayes classifier trained on the Greengenes 13_8 99% OTUs. (32,33)

Statistical analyses were performed in R (v3.5.1) and Qiime2 on rarefied data (depth of 2850 reads). Differential abundance and taxa dissimilarities across study groups was analysed using the Kruskal-Wallis ANOVA test with the R package *DESeq2*. All p-values were corrected for multiple comparisons by the Benjamini-Hochberg False Discovery Rate (FDR) procedure and considered significant when pFDR<0.05.

Fluorescence In Situ Hybridization (FISH)

Five micro sections of formalin fixed paraffin embedded tissues were cut ad placed on a slide. To visualize *E. faecalis*, we used a probe designed by Miacom Diagnostics for the fluorescence in situ hybridization (FISH) beacon-based technology. The probe was labelled with *E. faecalis* specific Alexa Fluor 647. Staining was performed per the manufacturer's protocol. We used confocal microscopy on Leica SP5II AOBS tandem scanner spectral confocal system on a DMI6000 microscope. The Leica microscope is controlled by LASAF software (version 2.8.3) with excitation/emission of 654/d755 nm. Objectives used were x20, NA 0.7 dry; x10, NA 0.4 dry, and x40, NA 1.25 oil (Leica).

Additional Experimental Antibiotics

Vancomycin, 4mg/kg (Med Vet International), ampicillin, 50g/kg (Med Vet International), spectinomycin, 500µg/mL (Henry Schein Animal Health), and streptomycin, 15µg/mL (Henry Schein Animal Health) were dissolved in 500mL of drinking water.

ABA-PEG20k-Pi20 (Pi-PEG)

The polyphosphate-containing polyethylene glycol based triblock co-polymer with defined ABA (hydrophilic-hydrophobic-hydrophilic) structure was synthesized according as previously described. (34) Pi-PEG was adjusted to pH of 7.4 with NaOH and added as a 1% solution to the drinking water of animals in select experiments. The pH was adjusted weekly to maintain pH 7.4.

Collagenase Assay of Selected Strains

Collagenase activity of selected strains was measured using fluorescein-labelled gelatine or fluorescein-labelled collagen (EnzChek® Gelatinase/Collagenase assay kit; Thermo Fisher Scientific, Waltham, Massachusetts, USA). Samples were suspended in TY medium (10g/L tryptone (EMD Chemicals, Gibbstown, NJ, USA) and 5g/L yeast extract (Sigma Life Science, St, Louis, MO, USA) were placed in 96-well plate as has been described previously. (9) In select experiments, Pi-PEG was added to designated samples to make a concentration of 1% in solution. pH of the Pi-PEG solution was adjusted to pH 7.4 with NaOH. Control 1% Pi-PEG was included in each run. Fluorescence (485/20 excitation, 528/20 emission) and optical density (OD) 600nm were measured at 0, 4 hours, 12 hours, and 24 hours. Zero-hour values were subtracted from overnight time points and samples were normalized for OD representing bacterial growth. To assess collagenase activity in Candida, the assay was run in according to the protocol above; however, the assay was allowed to continue for an additional 48 hours as peak change in OD occurs between 24 and 48 hours for this organism.

Metastatic Imaging

Distal colon and liver were harvested on POD21. Freshly harvested tissue was placed in a covered petri dish with 20µl normal saline to keep tissue moist and immediately analysed with the Leica SP5II AOBS tandem scanner spectral confocal system on a DMI6000 microscope for tomato fluorescence with excitation/emission of 400-600nm.

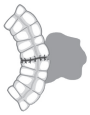
Quantitative PCR

For quantitative analysis of CT26 cells in the liver, a quantitative PCR was used QuantStudio™ 3 System (Thermo Fisher Scientific, USA). Calibration curve was created in each run with defined concentrations of CT26 DNA. A TaqMan primer-probe was designed in order to identify the luciferase (luc2) gene (Supplementary Table 2) (Primetime® Gene Expression Master Mix, Integrated DNA Technologies, Coralville, IA, USA). All threshold cycle (Ct) values of the luc2 gene were analysed by Quantstudio Design and Analysis Software (Thermo Fisher Scientific, USA) with an automatically generated threshold.

Stool Biobanking Study

Stool samples from patients 18 years or older undergoing an elective colon resection were collected between September 2017-2018 under IRB 17-0417 at the University of Chicago. An informed consent was obtained from all patients. Pre-operative stool samples were collected using the BioCollector at home stool sampling kit (BioCollective, Denver, CO, USA). These samples were collected prior to bowel preparation and shipped

directly to the laboratory. Intraoperative samples were collected by digital rectal exam after the induction of general anaesthesia.



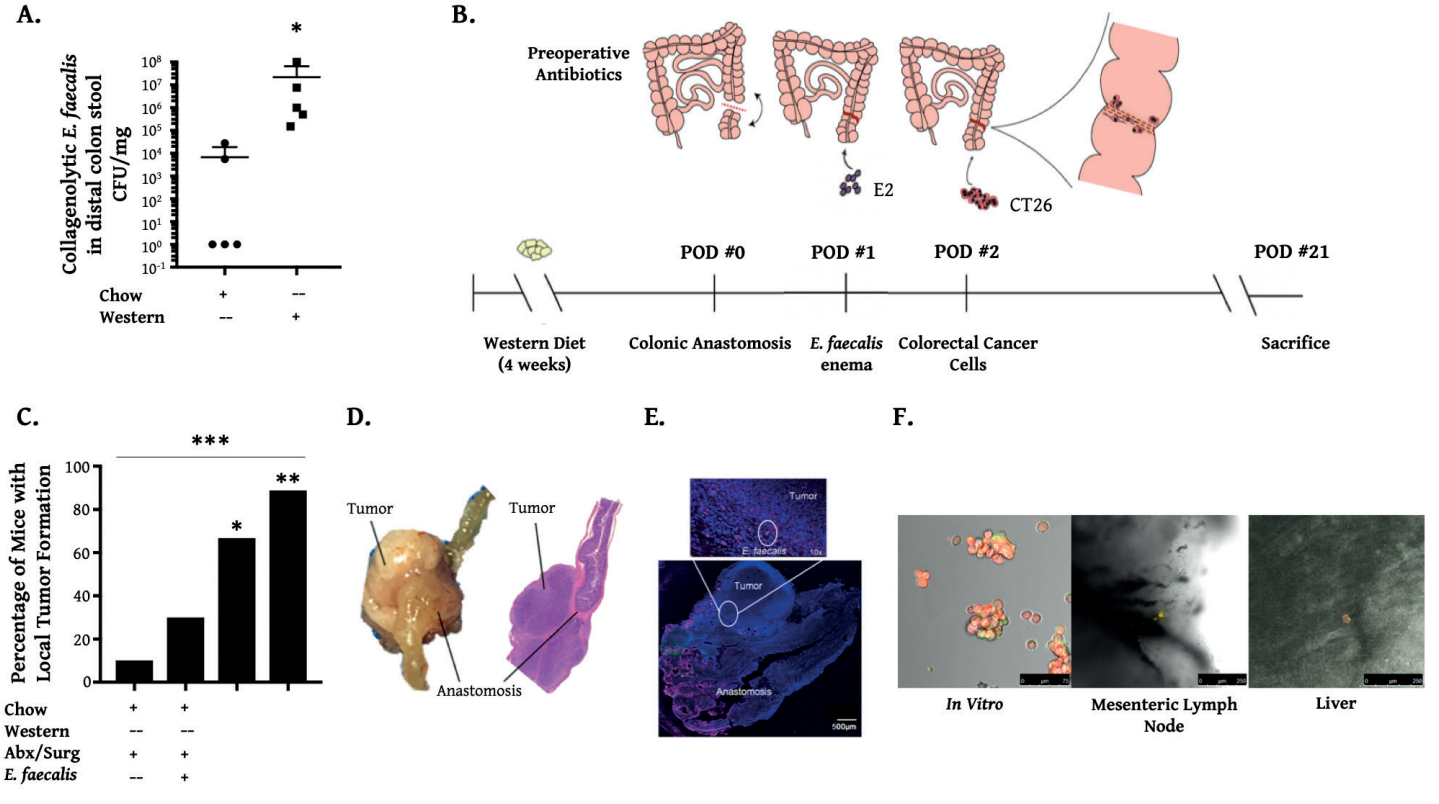
Statistical Analysis

Statistical analyses were performed using GraphPad Prism 8 software. Paired Student's t-tests were used for comparisons between two means. Mann-Whitney nonparametric test was used when there was no assumption of normal distribution. Fisher's exact test was used to describe two categorical values. Ordinary one-way ANOVA was used to compare the means of two or more samples. Kruskal-Wallis one-way ANOVA was used to compare two or more independent samples of equal or different samples sizes. 16S rRNA gene statistical analyses were described earlier in this section. Statistical significance was defined as p value <0.05. A mixed effects model was needed to allow for correlation between repeated measurements on a subset of individuals in the stool biobanking study. This model included main effect terms for weight group and timing of bowel preparation, as well as a group-by-time interaction. A statistically significant interaction indicated the magnitude of the before and after bowel preparation changes differed between normal and obese subjects.

RESULTS

Mice fed a Western Diet form extra-intestinal tumours following luminal exposure to collagenase producing *E. faecalis* and dispersed colon cancer cells

To determine the influence of a high fat/low fibre Western diet (WD) on the intestinal microbiota, BALB/c male mice were placed on four weeks of a polyunsaturated fatty acid diet consisting of 60% fat calories and no fibre versus a standard chow diet (std) consisting of 6.2% fat and 3.5% crude fibre (Supplementary Table 1). Results indicate that compared to std, WD-fed mice demonstrated a threefold higher colonization with collagenolytic *E. faecalis* in the distal colon ($2.18x10^7$ vs $6.6x10^3$ CFU/mg, *P < 0.05, Mann-Whitney test) (Figure 1A). Next, separate groups of mice were randomly assigned to 4 weeks of ad libitum feeding of either WD or std. As patients undergoing colon surgery receive both oral and parenteral prophylactic antibiotics, animals were also administered oral clindamycin and intramuscular cefoxitin and then subjected to a colorectal resection and subsequent anastomosis. To ensure equal exposure of anastomotic tissues to collagenolytic *E. faecalis* in both std and WD fed mice, a fixed volume and concentration of previously characterized collagenolytic rodent-derived strain of *E. faecalis* (strain E2) was delivered via enema on POD 1 (Figure 1B). Finally, to mimic the exposure of anastomotic tissues to shed cancer cells, an inevitable occurrence both during and after surgery, dispersed mouse colon cancer cells (CT26) were administered by enema on post-operative day 2 (POD 2).


A.
Collagenolytic E. faecalis in distal colon stool CFU/mg
Chow
Western
B.
Preoperative Antibiotics
E2
CT26
POD #0
POD #1
POD #2
POD #21
Western Diet (4 weeks)
Colonic Anastomosis
E. faecalis enema
Colorectal Cancer Cells
Sacrifice
C.
Percentage of Mice with Local Tumor Formation
Chow
Western
Abx/Surg
E. faecalis
D.
Tumor
Tumor
Anastomosis
E.
Tumor
E. faecalis
Tumor
Anastomosis
500μm
F.
In Vitro
Mesenteric Lymph Node
Liver

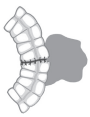
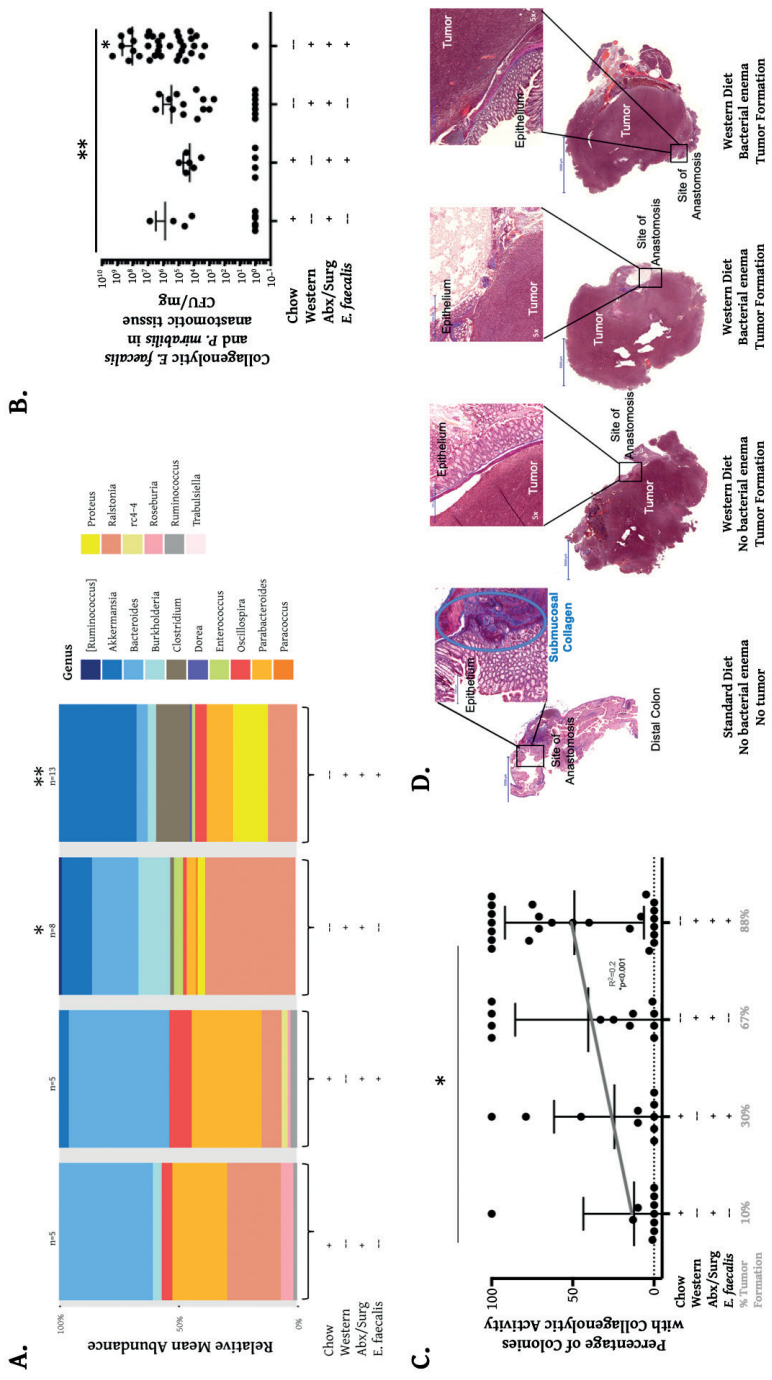


Figure 1 - Model of colorectal tumour formation in mice following surgery. **(A)** Mice fed a Western diet results in nearly three orders of magnitude increase ($6.6x10^3$ versus $2.18x10^7$ CFU/mg, n= 5 per group, *P < 0.05, Mann-Whitney test) in collagenolytic *E. faecalis* in the distal colon. **(B)** Mouse model of anastomotic colon tumour formation: mice are fed a Western diet (WD) or standard diet for four weeks, following which they are then administered oral and parenteral antibiotics 1 hour prior to surgery, and subject to a colorectal anastomosis. On post-operative day 1 (POD 1), anastomotic tissues were exposed to collagenolytic *E. faecalis* via enema. On post-operative day 2 (POD 2), anastomotic tissues were exposed to dispersed colorectal cancer cells (CT26) via enema. All animals are sacrificed on post-operative day 21 (POD 21). **(C)** Result of the necropsy demonstrated perianastomotic, extra-mucosal tumour formation in 67% of mice fed a WD compared to 10% of animals fed a standard chow diet (std) (n ≥ 10 per group, *P < 0.05, Fisher's exact test). Exposure of anastomotic tissues to *E. faecalis* increased tumour formation to 88% in WD fed mice compared to 30% in std fed mice (n ≥ 10 per group, **P < 0.05, Fisher's exact test). Differences in tumour formation among the groups was statistically significant. There were also significant differences in tumour incidence between control std diet fed mice and WD fed mice given *E. faecalis* (***P < 0.05, ordinary one-way ANOVA). **(D)** Representative gross tumours (left) and haematoxylin and eosin staining histology (right) demonstrated extra-mucosal tumour formation. **(E)** Fluorescence in situ hybridization (FISH) using *E. faecalis* specific probe Alexa Fluor 647 (pink) demonstrated *E. faecalis* colonization in anastomotic tissue and throughout the colonic tumour. **(F)** Representative image demonstrating tomato-labelled CT26 cells in mesenteric lymph nodes and liver tissue in WD fed mouse administered an *E. faecalis* enema.

Based on preliminary data demonstrating that tumours required approximately 21 days to form in this model, mice were observed and sacrificed on postoperative day 21 (POD 21). Necropsy found the presence of perianastomotic, extra-mucosal tumour formation in 67% (8 of 12) of WD fed mice compared to 10% (1 of 10) of std fed mice (*P < 0.05, Fisher's exact test). Exposure of anastomotic tissues to collagenolytic strains of *E. faecalis* via enema resulted in a significant increase in tumour formation (88% in WD fed mice versus 30% in std fed mice (**P < 0.05, Fisher's exact test). The percentage of mice with local tumour formation across all groups was statistically significant (***P < 0.05, ordinary one-way ANOVA) (Figure 1C, 1D). Necropsy indicated a well healed anastomosis as judged by our validated healing score of 1 (loose adhesion) or 2 (dense adhesion) on POD 21 in all groups of mice. (10) Finally, at sacrifice, the operative site in the colon where tumours were formed was opened and no mucosal tumours were observed upon gross inspection.

We next determined the anatomical distribution of *E. faecalis* at the site of anastomosis and within tumours using fluorescence in situ hybridization (FISH) beacon-based technology. Anastomotic colon tissue with peri-anastomotic tumour was incubated with an *E. faecalis* specific probe Alexa Fluor 647 (pink). Results indicated that *E. faecalis* organisms co-aggregated in peri-anastomotic tissues and were scattered throughout the tumour (Figure 1E).

In order to determine if intra-luminally introduced CT26 cells (via enema) penetrated beyond the extra-intestinal anastomotic tumour sites, we imaged for the presence of the dual labelled CT26 cells (luciferase and tomato) with the Leica sp2_photon tandem scanner. Analysis of the images revealed micrometastases in lymph node and liver in WD-fed mice inoculated with *E. faecalis* via enema (Figure 1F). Real-Time Polymerase Chain Reaction (RT-PCR) was used to quantify expression of the luc2 gene of the CT26 cells in livers of mice fed a WD versus std diet. Animals fed a WD had a trend toward an increase in the incidence of liver micrometastases compared to CD animals (40% vs 10%) (P = 0.1, Fisher's exact test) (Supplementary Figure 1).


A.
Relative Mean Abundance
100%
50%
0%
n=5
n=5
n=8
n=13
*
**
Genus
[Ruminococcus]
Akkermansia
Bacteroides
Burkholderia
Clostridium
Dorea
Enterococcus
Oscillospira
Parabacteroides
Paracoccus
Proteus
Ralstonia
rc4-4
Roseburia
Ruminococcus
Trabulsiella
Chow
Western
Abx/Surg
E. faecalis
B.
Collagenolytic E. faecalis and P. mirabilis in anastomotic tissue CFU/mg
**
*
Chow
Western
Abx/Surg
E. faecalis
C.
Percentage of Colonies with Collagenolytic Activity
100
50
0
*
R²=0.2
*p<0.001
Chow
Western
Abx/Surg
E. faecalis
% Tumor Formation
10%
30%
67%
88%
D.
Epithelium
Submucosal Collagen
Site of Anastomosis
Distal Colon
Standard Diet
No bacterial enema
No tumor
Epithelium
Tumor
5x
Site of Anastomosis
Tumor
Western Diet
No bacterial enema
Tumor Formation
Epithelium
Tumor
5x
Tumor
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Western Diet
Bacterial enema
Tumor Formation

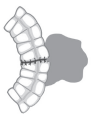


Figure 2 - Tumour formation is associated with collagenolytic *E. faecalis* and *P. mirabilis* in anastomotic tissues. (A) 16S rRNA taxonomic proportions demonstrated that mice fed a WD harbour a significant increase in the proportion of *Proteus,* and a significant increase in both *Proteus* and *Enterococcus* when administered *E. faecalis* compared to std fed mice (*P < 0.05). **(B)** Colonization of anastomotic tissues with collagenolytic *E. faecalis* or *P. mirabilis* was increased in WD-fed mice following *E. faecalis* enema ($8.8x10^7$ vs $2.9x10^5$ CFU/mg, n ≥ 10 per group, *P < 0.05, Mann-Whitney test). Colonization of collagenolytic *E. faecalis* and *P. mirabilis* was statistically significant among all groups (**P < 0.05, Kruskal-Wallis test). **(C)** Analysis of the percentage of bacterial colonies with collagenase activity compared to total bacteria showed a linear correlation between incidence of local tumour formation and collagenolytic burden in the anastomotic tissues (n ≥ 10 per group, *P < 0.05, ordinary one-way ANOVA, R^2=0.2). **(D)** Tri-chrome collagen stain demonstrated a decrease in submucosal collagen fibres (blue) in tumour forming mice, with minimal collagen present between the tumour and adjacent mucosa.

Anastomotic *E. faecalis* or *P. mirabilis* colonization significantly correlates with tumour formation

The compositional changes of the anastomotic microbiome that associate with tumour formation were determined using 16S rRNA gene amplicon sequencing, which indicated that WD fed mice had a significant increase in the proportion of the bacterial genera *Proteus* (**pFDR=0.005), *Akkermansia* (**pFDR=0.001) and *Trabulsiella* (**pFDR=$1.81x10^{-11}$), but a decrease in *Bacteroides* (*pFDR=0.027), *Roseburia* (**pFDR=$5.82x10^{-15}$) and *Ruminococcus* (*pFDR=0.048) compared to std diet. In the subset of animals in which *E. faecalis* was introduced, assessed by relative mean abundance, there was a bloom in the genera *Proteus* (**pFDR=$9.52x10^{-14}$), *Enterococcus* (**pFDR=$9.52x10^{-14}$), *Trabulsiella* (**pFDR=0.008) *Akkermansia* (*pFDR=0.033) and *Clostridium* (*pFDR=0.012), while genus *Roseburia* proportionally decreased (**pFDR=$2.98x10^{-5}$) in WD fed mice. (Figure 2A). Absolute quantity (pg/ml) of *Enterococcus* and *Proteus* were calculated using quantitative PCR to determine total bacterial load and relative absolute abundance from 16S analysis (Supplementary Table 3).

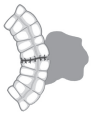
Our previous work demonstrated that both *P. mirabilis* and *E. faecalis* express a collagenolytic phenotype capable of disrupting the integrity of anastomotic tissues. Given the observation that both *Proteus* and *Enterococcus* were increased in WD fed mice (which displayed the highest incidence of tumour formation). Therefore, we next examined the bacterial collagenase activity of *E. faecalis* or *P. mirabilis* among the groups. (9) There was a significant difference in colonization density of collagenolytic *E. faecalis* and *P. mirabilis* between all groups (**P < 0.05, Kruskal-Wallis test) (Figure 2B). An approximately three orders of magnitude increase in the density of collagenolytic bacteria was observed between WD fed mice compared to std fed mice ($8.8x10^7$ vs $2.9x10^5$ CFU/mg,*P < 0.05, Mann-Whitney test) when exposed to the indicated elements of the model (*E. faecalis* enema, antibiotics, surgery). Further analysis of the percentage of total bacterial colonies with collagenolytic activity compared to total bacteria present in the anastomotic tissue revealed a linear correlation between percentage

of tumour formation and collagenase activity when collagenolytic *E. faecalis* and *P. mirabilis* activities were combined (*P < 0.05, ordinary one-way ANOVA, R^2=0.2) (Figure 2C). Histological analysis using trichrome staining for collagen demonstrated a reduction in submucosal collagen in mice with local tumour formation, consistent with increased collagenase activity. (Figure 2D).

Antibiotics directed at eliminating collagenolytic pathobiota in this model failed to reduce tumour formation but selected for the emergence of fungal collagenase-producing microorganisms

To validate its role in tumour formation in this model, *E. faecalis* eradication was attempted in WD fed mice using oral vancomycin treatment both one day prior to surgery and throughout the study period (21 days). To our surprise, there was no difference in tumour formation (50% vs 40%, P=0.17, Fisher's exact test) or tumour-associated collagenolytic microbes ($2.9x10^5$ vs $1.8x10^6$ CFU/mg, P=0.2, Mann-Whitney test) between animals receiving vancomycin versus controls (Figure 3A). However, vancomycin treatment resulted in a significant disruption to the bacterial community, characterized by reduced relative abundance of all phyla that predominated (Firmicutes (**pFDR=$1.11x10^{-7}$), Verrucomicrobia (**pFDR=$1.51x10^{-4}$) and Bacteroidetes (**pFDR=$5.18x10^{-4}$)), except for an increase of Proteobacteria (**pFDR= $1.11x10^{-7}$) (Figure 3B). Compared to the non-vancomycin treated controls, a proportional decrease in *Bacteroides* (**pFDR=$1.07x10^{-20}$), *Clostridium* (**pFDR=$2.31x10^{-4}$), *Dorea* (**pFDR=$3.09x10^{-9}$), *Akkermansia* (**pFDR=$8.00x10^{-7}$), and *Parabacteroides* (**pFDR=$5.18x10^{-5}$) was observed in vancomycin treated mice, and an increase in *Sutterella* (**pFDR=$1.55x10^{-5}$) and *Proteus* (*pFDR=0.016) was observed (Figure 3C). Absolute quantity (pg/ml) of *Enterococcus* and *Proteus* were calculated using quantitative PCR to determine total bacterial load and relative absolute abundance from 16S analysis (Supplementary Table 3).

In order to address the possibility that *E. faecalis* and *P. mirabilis* persisted in the presence of vancomycin, reiterative experiments were repeated using a combination of ampicillin, spectinomycin and streptomycin, agents specifically targeting these two organisms. Similarly, no difference in tumour formation was observed in non-antibiotic treated versus the penta-antibiotic treated mice (75% vs 60%, P=0.6, Fisher's exact test). Interestingly, although bacterial culture demonstrated that the chosen antibiotics did indeed eliminate tumour-associated collagenolytic *E. faecalis* and *P. mirabilis* ($9.4x10^6$ vs 0 CFU/mg, *P < 0.05, Mann-Whitney test) (Figure 3D), we observed an emergence of *Candida parapsilosis*, a highly collagenolytic fungal strain. Its collagenolytic activity was assessed by examining its ability to degrade both collagen 1 and collagen 4 (Figure 3E), key collagen subtypes important to maintain anastomotic integrity.



Pi-PEG, a co-polymer with known bacterial collagenase suppressive activity, significantly decreases tumour formation in this model, while maintaining microbial diversity

Phosphate is an environmental cue for many pathogenic bacteria; it both supports their growth and suppresses virulence via highly conserved phosphosensory/phosphoregulatory elements that connect to quorum sensing circuits. (34) We have previously synthesized *de novo* a phosphate carrier compound ABA-PEG20k-Pi20 (Pi-PEG) and determined that it suppresses bacterial collagenase production *in vitro* while preserving anastomotic integrity *in vivo*. (10,34) Here we tested the ability of a 1% solution of Pi-PEG provided in the drinking water of mice to prevent tumour formation in this model. Mice were administered Pi-PEG for three days prior to anastomotic surgery and for the remainder of the study period (21 days). Given that WD fed mice treated with prophylactic antibiotics, surgery and *E. faecalis* administration developed the highest incidence of tumours in this (88%) in this model, these were the conditions used for the control group. Results indicated that mice drinking the Pi-PEG solutions demonstrated a 57% reduction in tumour formation (66% vs 9%, *P < 0.05, Fisher's exact test) compared to controls (WD + Abx) and a threefold reduction in tumour associated collagenolytic *E. faecalis* and *P. mirabilis* ($1.1x10^4$ vs $5.6x10^1$ CFU/mg, **P < 0.05, Mann-Whitney) (Figure 4A). Mice drinking Pi-PEG showed no significant difference in the proportion of bacterial phyla compared to controls (Figure 4B). However, Pi-PEG consumption resulted in a significant decrease in the proportion of *Proteus* (**pFDR=$8.84x10^{-4}$) in Pi-PEG treated mice compared to controls (Figure 4C). Absolute quantity (pg/ml) of *Enterococcus* and *Proteus* were calculated using quantitative PCR to determine total bacterial load and relative absolute abundance from 16S analysis (Supplementary Table 3). Following a 20 hour incubation period, Pi-PEG *in vitro* suppressed the rate of collagenase production of both *E. faecalis* ($1.2x10^3$ vs $0.19x10^3$ RFU/time *P < 0.05, paired student's t test) and *P. mirabilis* ($1.08x10^3$ vs $0.25x10^3$ RFU/time, **P < 0.05, paired student's t test) (Figure 4D). To test the applicability of Pi-PEG on the suppression of collagenase activity of *Candida parapsilosis*, degradation of collagen 1 and 4 were measured after incubating the strain with the highest collagenase activity with and without 1% Pi-PEG. Pi-PEG reduced the ability of *C. parapsilosis* to degrade both collagen 1 ($15.7x10^3$ vs $2.4x10^3$RFUs) and collagen 4 ($5.5x10^3$ vs $0.99x10^3$ RFUs) (Supplementary Figure 2). FISH staining using the *E. faecalis* specific probe Alexa Fluor 647 (pink) demonstrated that Pi-PEG eliminated *E. faecalis* in the tumour environment (Figure 4E). Five control std animals treated with Pi-PEG did not develop peri-anastomotic tumours, and there was no colonization of collagenolytic organisms within the anastomotic tissue. Finally, in all mice examined treated with Pi-PEG mice (n=4), CT26 tumour cells were not observed in the liver.

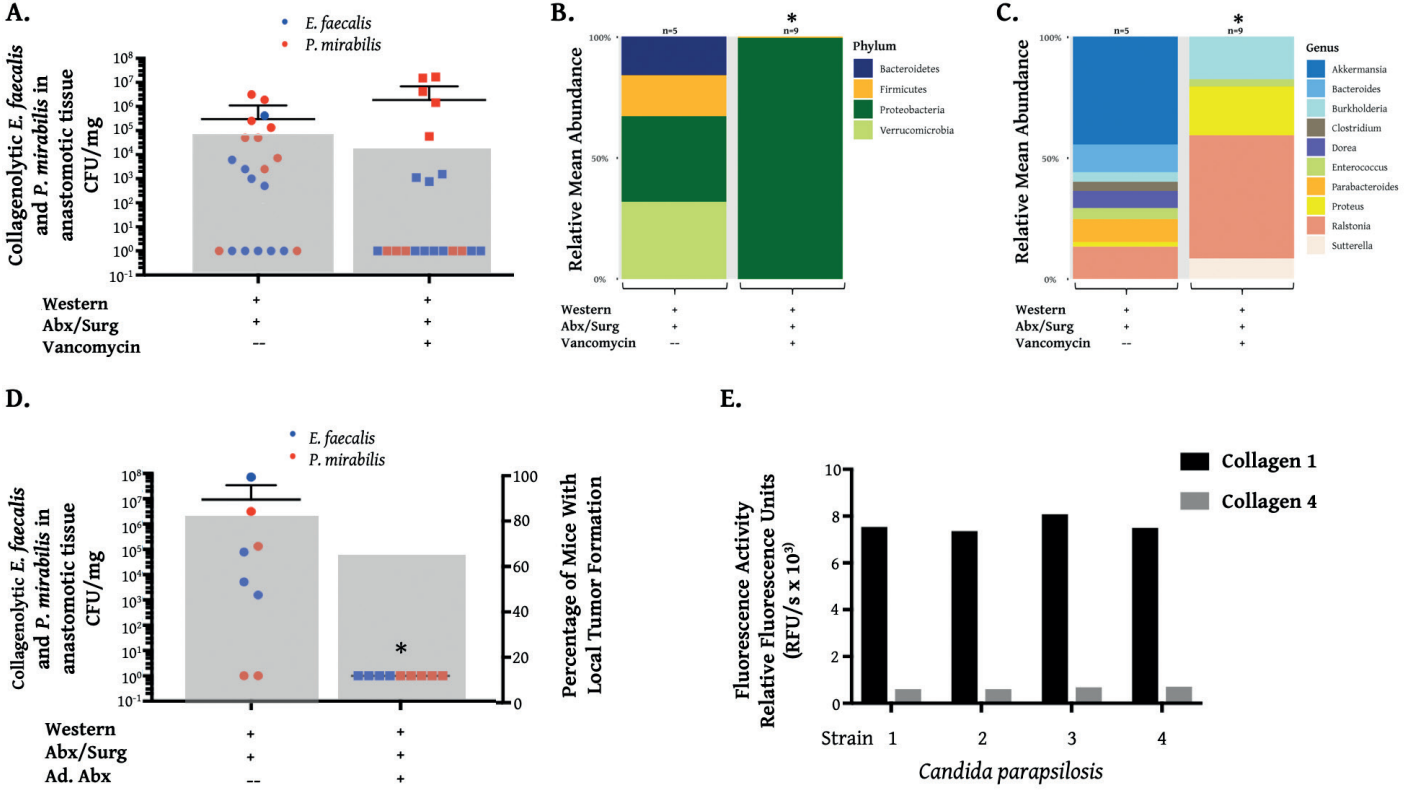

A.
E. faecalis
P. mirabilis
Collagenolytic E. faecalis and P. mirabilis in anastomotic tissue CFU/mg
Western
Abx/Surg
Vancomycin
B.
Relative Mean Abundance
Phylum
Bacteroidetes
Firmicutes
Proteobacteria
Verrucomicrobia
n=5
n=9
C.
Genus
Akkermansia
Bacteroides
Burkholderia
Clostridium
Dorea
Enterococcus
Parabacteroides
Proteus
Ralstonia
Sutterella
D.
Ad. Abx
Percentage of Mice With Local Tumor Formation
E.
Collagen 1
Collagen 4
Fluorescence Activity Relative Fluorescence Units (RFU/s x 10³)
Strain 1 2 3 4
Candida parapsilosis

Figure 3 - Broadening the antibiotic coverage in the model resulted in the emergence of additional collagenase producing saprophytic organism, but did not prevent tumour formation (**A**) Vancomycin failed to reduce both collagenolytic *E. faecalis* and *P. mirabilis* ($2.9x10^5$ vs $1.8x10^6$ CFU/mg, n= 10 per group, P =0.2, Mann-Whitney test) and tumour formation (50% vs 40%, n=10 per group, P =0.17, Fisher's exact test). (**B/C**) Treatment with vancomycin significantly disrupted organization on phylum level by diminution of Firmicutes (**pFDR= $1.11x10^{-7}$), Verrucomicrobia (**pFDR=$1.51x10^{-4}$) and Bacteroidetes (**pFDR=$5.18x10^{-4}$) and expansion of Proteobacteria (**pFDR= $1.11x10^{-7}$). On the genus level, vancomycin treatment caused a proportional decrease in *Bacteroides* (**pFDR=$1.07x10^{-20}$), *Clostridium* (**pFDR=$2.31x10^{-4}$), *Dorea* (**pFDR=$3.09x10^{-9}$), *Akkermansia* (**pFDR=$8.00x10^{-7}$), and *Parabacteroides* (**pFDR=$5.18x10^{-5}$) compared to non-vancomycin treated mice, and showed an increase in *Sutterella* (**pFDR= $1.55x10^{-5}$) and *Proteus* (*pFDR=0.016) (**D,E**) Elimination of collagenolytic *E. faecalis* and *P. mirabilis* ($9.4x10^6$ vs 0 CFU/mg, n=5 per group,*P < 0.05, Mann-Whitney test) using a multiple antibiotic cocktail failed to prevent tumour formation (75% vs 60%, n=5 per group, P =0.6, Fisher's exact test) but led to the emergence of collagenolytic *Candida parapsilosis* which displayed a high capacity to degrade collagen 1 and moderate ability to degrade collagen 4.

Collagenase producing organisms in the colon vary among subsets of patients undergoing colon surgery: the role of obesity

To determine the clinical relevance of our findings, we surveyed patients undergoing colon resection or rectal resection for their ability to harbour collagenolytic organisms under Institutional Review Board (IRB 17-0417) protocol approved by the University of Chicago. Preoperative stool was collected prior to initiation of routine bowel antisepsis (i.e. mechanical bowel preparation-MBP) and oral antibiotics (neomycin and metronidazole) and intraoperatively by digital rectal exam following induction of general anaesthesia. As would be predicted given the spectrum of antibiotics used, MBP + prophylactic parenteral antibiotics reduced the total quantity of collagenase producing microbes in patients (pre-MBP $3.56x10^4$ vs post-MBP $2.08x10^4$ RFU; pre-MBP n=48, post-MBP n=29,*P < 0.05, unpaired student's t test) (Figure 5A). However when patients were stratified by BMI, the quantity of collagenase producing microbes in patients with obesity (BMI of >29) actually increased following MBP ($3.2x10^4$ vs $5.6x10^4$ RFU; pre-MBP n=14, post-MBP n=5); whereas MBP decreased collagenolytic bacteria in normal weight patients ($3.9x10^4$ vs $1.5x10^4$ RFU; pre-MBP n=34, post-MBP=24). Mixed effect modelling considering repeated measurements of a subset of individuals and group by time interactions demonstrated a *P < 0.05 indicating a difference in response to bowel preparation between normal and obese patients (Figure 5B).

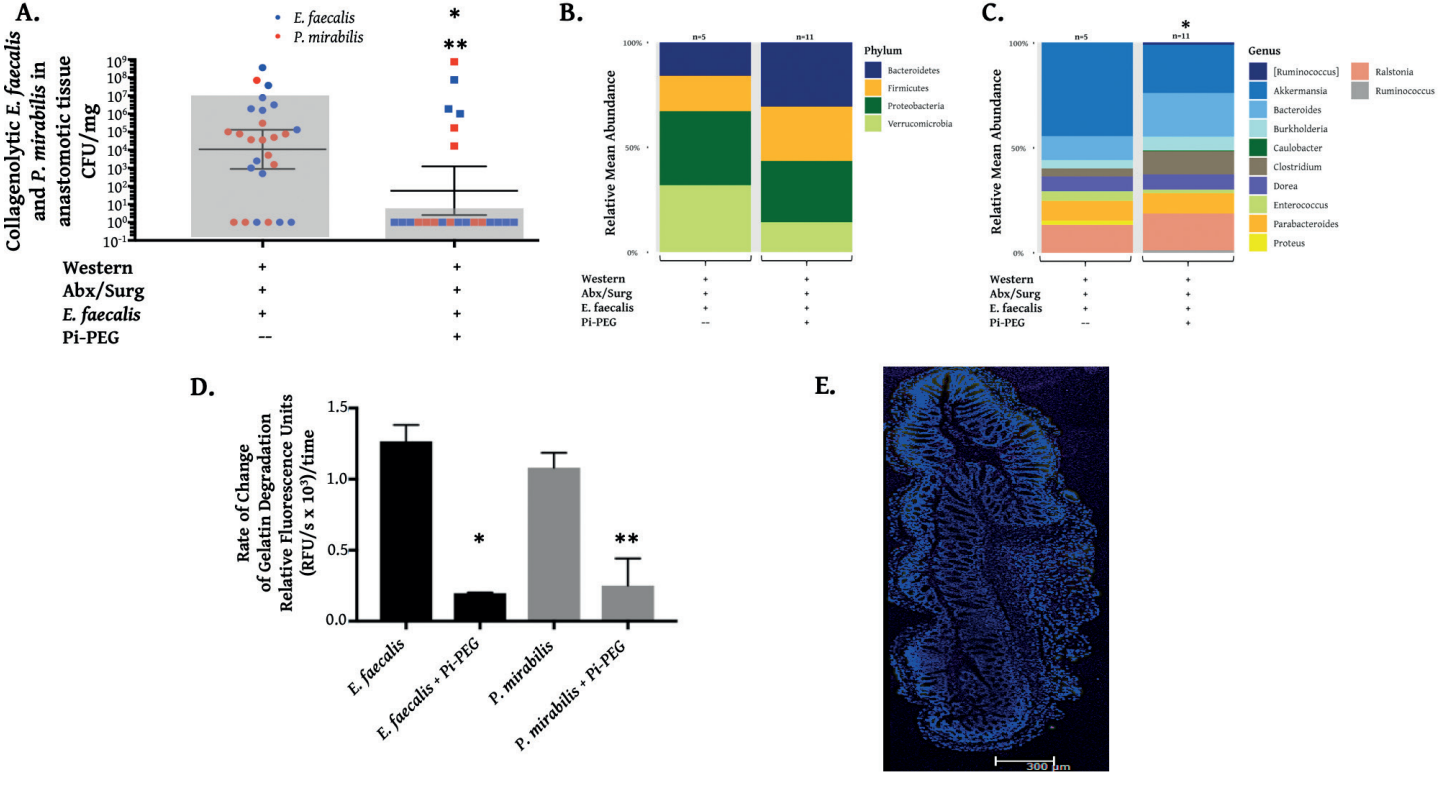

A.
E. faecalis
P. mirabilis
Collagenolytic E. faecalis and P. mirabilis in anastomotic tissue CFU/mg
Western
Abx/Surg
E. faecalis
Pi-PEG
B.
Relative Mean Abundance
Phylum
Bacteroidetes
Firmicutes
Proteobacteria
Verrucomicrobia
C.
Genus
[Ruminococcus]
Akkermansia
Bacteroides
Burkholderia
Caulobacter
Clostridium
Dorea
Enterococcus
Parabacteroides
Proteus
Ralstonia
Ruminococcus
D.
Rate of Change of Gelatin Degradation Relative Fluorescence Units (RFU/s x 10³)/time
E. faecalis
E. faecalis + Pi-PEG
P. mirabilis
P. mirabilis + Pi-PEG
E.
300 µm

Figure 4 - Pi-PEG suppresses collagenase production *in vitro* and decreases tumour formation *in vivo*. (**A**) Mice drinking Pi-PEG displayed a three order magnitude reduction in collagenolytic *E. faecalis* and *P. mirabilis* ($1.1x10^4$ vs $5.6x10^1$ CFU/mg, n=15 per group, **P < 0.05, Mann-Whitney) on anastomotic tissues and a 57% reduction in tumour formation (66% vs 9%, n=15 per group, *P < 0.05, Fisher's exact test). (**B/C**) 16S rRNA gene amplicon analysis of anastomotic tissue demonstrating no effect of Pi-PEG at the phylum level, but a proportional decrease in the genus *Proteus* (**pFDR= $8.84x10^{-4}$). (**D**) In vitro collagenase assay demonstrating Pi-PEG decreases the rate of collagenase production over a 20 hour incubation for both *E. faecalis* ($1.2x10^3$ vs $0.19x10^3$ RFU/time ,*P < 0.05, Paired student's t test) and *P. mirabilis* ($1.08x10^3$ vs $0.25x10^3$ RFU/time, **P < 0.05, Paired student's t test). (**E**) Fluorescence in situ hybridization (FISH) with an *E. faecalis* specific probe Alexa Fluor 647 (pink) demonstrated the absence of *E. faecalis* in Pi-PEG treated mouse anastomotic tissue.

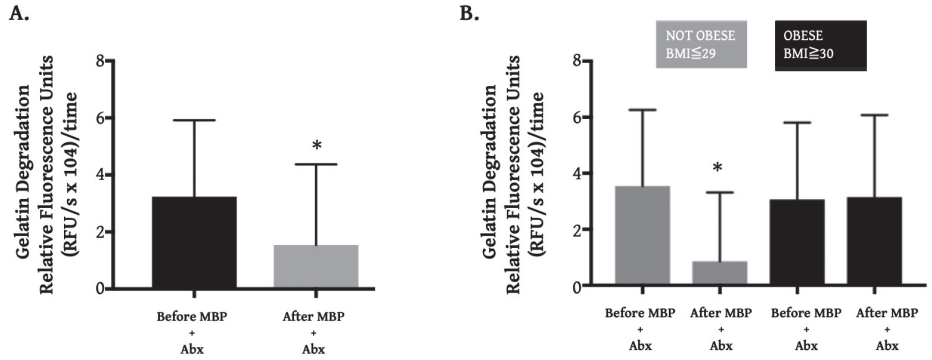


Figure 5 - Effect of mechanical bowel prep (MBP= purgative cleansing) and oral and parenteral antibiotics (Abx) on collagenase producing organisms. (**A**) MBP reduced the quantity collagenase producing microbes in patients when comparing pre-MBP + Abx samples to post MBP + Abx samples (pre-MBP $3.56x10^4$ vs post-MBP $2.08x10^4$ RFU; pre-MBP n=48, post-MBP n=30,*P < 0.05, unpaired student's t test) (**B**) Results indicating the failure of MBP + Abx to eliminate collagenase producing organisms in obese patients ($3.2x10^4$ vs $5.6x10^4$ RFU; pre-MBP n=14, post-MBP n=5). MBP + Abx led to a two-fold reduction in collagenase producing organisms in non-obese patients ($3.9x10^4$ vs $1.5x10^4$ RFU; pre-MBP n=34, post-MBP=24). Mixed effect modelling considering repeated measurements of a subset of individuals and group by time interactions demonstrated a *P < 0.05 indicating a difference in response to bowel preparation between normal and obese patients

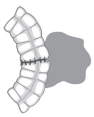
DISCUSSION

Technical advances and treatment options have improved outcomes for patients with colorectal cancer. Yet, even when a pathologically confirmed complete resection has presumably been achieved, both local and distant metastases can occur; the underlying mechanisms remain elusive. Data from the present study demonstrate that viable cancer cells in the colon lumen can penetrate intact anastomotic tissues and form tumours in a clinically relevant environmental context. We hypothesize that colonization by collagenolytic bacteria of anastomotic tissues disrupts the healing intestine resulting in increased permeability and transmigration of cancer cells. The finding that collagenolytic bacteria are enriched in the colon of mice fed a high fat diet is especially intriguing given the known role of collagenolytic bacteria in the

pathogenesis of anastomotic leak coupled with the knowledge that the incidence of recurrent CRC is associated with both anastomotic leak and consumption of a high fat, Western diet. (9,13,21) Although the precise role and mechanisms of shed cancer cells leading to a local recurrence or possibly distant disease following surgery is likely multifactorial and will require further study, the model herein described suggests that exposure of mice to the multiple elements that encompass the human disease (i.e. an intestinal wound, antibiotics, Western diet, etc.) may be necessary.

While understanding the role of the microbiome in the etiopathogenesis of CRC is critically important, its role in disease recurrence following surgery has received little attention. Patients routinely receive both oral and parenteral antibiotics prior to colon cancer surgery although the type, route, and dosing of antibiotics is highly variable among practitioners. Yet the use of a broad bacterial killing strategy to achieve intestinal antisepsis prior to surgery does not consider the collateral damage it might inflict on the normal microbiota and increased pathogenic bacterial or fungal phenotypes that might emerge from this practice. For example, here we attempted to decontaminate mice of collagenolytic bacteria prior to surgery with multiple antibiotics to target resistant *E. faecalis* and *P. mirabilis*. This approach failed to reduce tumour formation. These results are consistent with enhanced tumour progression after long-term treatment with an antibiotic cocktail and depletion of intestinal microbiome in a commonly used murine model of familial adenomatous polyposis, $Apc^{Min/}$ model. The antibiotic treated mice in that study had a reduction in mucus producing goblet cells which are known to be protective to the colonic epithelium and a vital nutrient source for the microbiome. (35-37) Our attempt to indiscriminately eliminate all collagenolytic pathogens resulted in the subsequent overgrowth of *Candida parapsilosis*, which itself is highly collagenolytic. The ability of this pathogen to secrete collagenase that degrades collagen 1 and 4 has not previously been described among the known secreted enzymes; however, the *Candida* genus is known to secrete Secreted aspartic proteases (Saps) which can degrade certain cellular substrates such as collagen. (38) Others have shown that *Candida parapsilosis* can emerge when patients have received multiple antibiotics. (39)

Given that the standard approach to eliminate all potential pathogens resulted in severe dysbiosis and the emergence of an opportunistic pathogen with a similar virulence to *E. faecalis* and *P. mirabilis* (i.e. *Candida parapsilosis*), we employed a more targeted approach using Pi-PEG. We have previously demonstrated that a wide variety of microbes express enhanced virulence in a phosphate-depleted environment. Phosphate covalently linked to PEG allows it to distribute along the entire mouse intestine. Work from our laboratory has established that Pi-PEG, while not affecting bacterial growth, increases the local phosphate concentration and consequently



decreases microbial virulence. (40-43) Here we found that Pi-PEG inhibits collagenase production from *E. faecalis* and *P. mirabilis* and protects mice from tumour formation. We also found that Pi-PEG does not disrupt the intestinal microbiome community or promote proliferation of other virulent pathogens, providing an advantage over conventional antibiotics. While further confirmatory studies are needed, we theorize that the efficacy of Pi-PEG to reduce tumour formation in this model lies in its ability to preserve the integrity of the normal microbiota and suppress commensals from expressing a virulent collagenolytic phenotype.

There are several limitations to the present study and the model. While the presence of collagenolytic organisms are strongly associated with tumour formation, and in their absence, no malignant disease developed, we were unable to prove causality. This would require selective elimination of these two species within the model or alternatively targeted gene knockout to selectively disable collagenase activity. While technically feasible using gene editing, these studies are methodologically complex and beyond the scope of the present study. Alternatively, these experiments could be repeated using this model in germ-free mice colonized with collagenase producing organisms and compared to strain-specific mutants deleted of collagenase genes. While this was considered, wound healing in germ free mice is not normal and performing surgery in germ-free mice can be problematic, especially in the colon. (44) Additionally, use of Pi-PEG compound alone is insufficient to establish causality between bacterial collagenase production and tumour formation, as this agent may have had other effects beyond its ability to suppress bacterial collagenase. These include, for example, its ability to enhance and even preserve mucus production and maintain tight junction integrity. (10) Conclusions regarding the metastatic potential beyond the confines of the perianastomotic tissues are limited as this was not the focus of the study.

Overall the findings in this model beg a real need for a more complete understanding of how the combined effects of diet, antibiotic use, and surgery influence microbiome structure, membership and function. In the context of colon cancer surgery, understanding how these factors, either permissively or actively, allow shed colon cancer cells to penetrate a surgically intact anastomotic tissues may be important to elucidate the process of colon cancer recurrence following curative resection. The finding that mechanical bowel preparation, including purgative cleansing and oral and parenteral antibiotics, successfully decontaminated the colon of collagenolytic pathobiota in some but not other patients may provide a clue as to why a subset of patients develop recurrence. These preliminary observations, if confirmed in larger powered studies, might have significant clinical implications in the preoperative management of patients. Finally, the implications of the present study

on cancer recurrence rates following curative colorectal cancer surgery suggests that longitudinal tracking of the colon microbiota using the microbiome and metabolome profiling may inform dietary recommendations and bowel preparations needed to reduced loco-regional recurrence rates.

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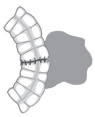

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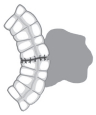
SUPPLEMENTARY FILES

Supplementary Table 1 – Nutritional content of standard, murine chow diet compared to Western diet based on vendor's information.

	Chow	Western
Fatty acids, g/kg		
Total saturated	9	141
Total monounsaturated	13	162
Total polyunsaturated	34	40.2
Fat, %	6.2	36
Fibre		
Crude Fibre, **%**	3.5	0
Neutral detergent fibre	14.7	0
Crude protein, %	18.6	20.5
Carbohydrates, %	44.2	35.7
Simple sugars	3	22
Complex sugars	41	14
Calories, kcal/g		
Calories from protein	0.24	0.82
Calories from fat	0.18	3.24
Calories from carbohydrate	0.58	1.43
Minerals, g/kg		
Calcium	10	5.6
Chloride	4	0.86
Phosphorus	7	5.8
Sodium	2	0.57
Potassium	6	5.6
Magnesium	2	0.49
Zinc	0.07	0.022
Manganese	0.1	0.047
Copper	0.015	0.004
Iodine	0.006	0.003
Iron	0.2	0.05
Selenium	0.00023	0.00021
Amino Acids, g/kg		
Aspartic Acid	14	12.8
Glutamic Acid	34	40.6
Alanine	11	5.3
Glycine	8	4.9
Threonine	7	8.7
Proline	16	20.5
Serine	11	11.4
Leucine	18	16.6



Supplementary Table 1 – Nutritional content of standard, murine chow diet compared to Western diet based on vendor's information.

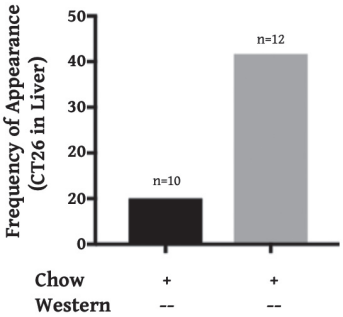
	Chow	**Western**
Isoleucine	8	11
Valine	9	13
Phenylalanine	10	8.9
Tyrosine	6	11.4
Methionine	4	7.1
Cystine	3	0.6
Lysine	9	14.8
Histidine	4	5.5
Arginine	10	7.3
Tryptophan	2	2.2
Vitamins, mg/kg		
Vitamin K3 (menadione)	50	0.52
Vitamin B1 (thiamin)	17	3
Vitamin B2 (riboflavin)	15	2.3
Niacin (nicotinic acid)	70	15
Vitamin B6 (pyridoxine)	18	4.1
Pantothenic Acid	33	5.5
Vitamin B12 (cyanocobalamin)	0.08	0.04
Folate	4	0.75
Choline	1200	1148
Vitamins, IU/kg		
Vitamin A	15000	3162
Vitamin E	110	25.7

Supplementary Table 2 - Luc2 gene sequences (Taqman).

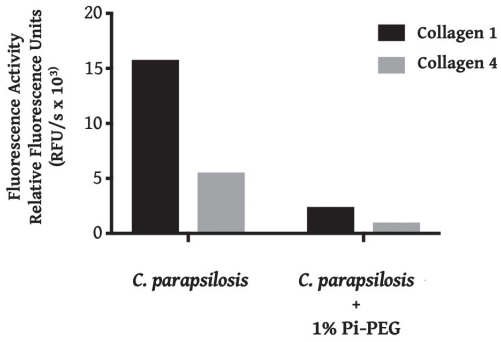
Luc2	**Sequence**
Forward	5'-GTGGTGTGCAGCGAGAATAG-3'
Probe	5'-TTGCAGTTCTTCATGCCCGTGTTG-3'
Reverse	5'- CGCTCGTTGTAGATGTCGTTAG-3'

Supplementary Table 3 - Mean absolute amount of the genera *Enterococcus* and *Proteus* in obtained samples (pg/ml) based on percentages of 16S rRNA gene sequencing and qPCR of Eubacteria primers (F: 5'-TCCTACGGGAGGCAGCAGT-3' and R: 5'-GGACTACCAGGGTATCTAATCCTGTT-3')

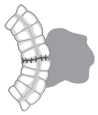
Group	*Enterococcus*	*Proteus*
Std + Abx	81	0
Std + Abx + EF	0	0
WD + Abx	465	228
WD + Abx + EF	265	627
WD + PiPEG	42	2
WD + Vancomycin	390	9549



Supplementary Figure 1: Western diet fed mice had a non-statistically significant increase in liver micrometastases compared to chow fed mice (40% vs 10%) (P =0.1, Fischer exact test).

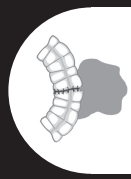


Supplementary Figure 2: Incubation of *Candida parapsilosis* in TY with 1% Pi-PEG reduced the ability of the fungus to degrade collagen 1 ($15.7x10^3$ vs $2.4x10^3$RFUs) and collagen 4 ($5.5x10^3$ vs $0.99x10^3$ RFUs).



WESTERN DIET

The term Western diet is used for the diet consumed in western countries. Typically, it contains high amounts of (satured) fat, sugars and carbohydrates but very low amounts of fiber. This type of diet has been suggested to have a major impact on human health, probably partly due to its influence on the gut microbiota. It is also positively correlated with an elevated incidence of obesity.



CHAPTER 8

SUMMARY, GENERAL DISCUSSION AND CONCLUSION

SUMMARY AND GENERAL DISCUSSION

Colorectal cancer has one of the highest incidences of all malignancies, with every year over a million new diagnosed patients. (1) Surgical resection of the tumour with the creation of an anastomosis is the standard treatment of care, often combined with neoadjuvant chemotherapy and/or radiation therapy. The most feared complication after this type of surgery is anastomotic leakage (AL).

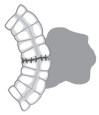
Despite perfectionated surgical techniques and the reduction of known risk factors, the incidence of AL has barely decreased over the past decades. Part of the problem is that the mechanism(s) behind the development of the leakages are just partly elucidated. In order to establish adequate prevention of AL to occur, first these mechanisms should be further explored.

This thesis describes the quest to elucidate the mechanisms behind AL. It uses new techniques to elaborate on a long-ignored contributor to AL and its consequences: the intestinal microbiota. It describes how the bacterial composition at the time of surgery plays a role in the development of AL. This may be in combination with a less favourable status of the patient's gene expression in wound healing related genes. Furthermore, this thesis shows that not only the bacterial composition, but also the virulence factors of bacteria and other microorganisms play a role in AL and the tumour recurrence that is associated with AL.

Chapter 2 and 3 describe the use of 16S rRNA gene sequencing (16S analysis) on samples obtained during colorectal surgery with the construction of an anastomosis. The first of these two chapters is a pilot study with non-C-seal samples, samples from patients that were randomized for standard care (an anastomosis without the use of the C-seal). The C-seal is a biodegradable intraluminal sheet designed for the protection of the colorectal anastomosis. This study showed that the samples we obtained during the C-seal trial were suitable for 16S analysis. Fifteen of the 16 samples had sufficient isolated DNA and subsequent analysis could be done. We used the V3–V4 region of the 16S rRNA gene to identify the bacteria present in the samples up to species level.

Although body mass index (BMI) was slightly higher in the group of patients with AL, this was not a significant or an independent factor for AL in this study group. Neither were other patients' characteristics. In AL, the bacterial composition did not show a significant difference compared to the control group, but there was a significantly lower microbial diversity, mostly accounted for by the bacterial families *Lachnospiraceae* and *Bacteroidaceae.*

In the subsequent **Chapter 3**, the results of a larger cohort are shown, with the addition of samples from patients that received a C-seal. When we analysed the



complete cohort of **Chapter 3** no differences in bacterial composition were found between AL and non-AL patients when the 118 samples were analysed together; only the *Blautia* genus was more abundant among AL patients. Although this was surprising, the lack of differences in the overall group could be attributed to the patients with a C-seal. The microbial composition of these samples did not differ significantly, however, in the C-seal trial, we previously found a trend to more AL in C-seal patients than in non-C-seal patients. (2) This suggests that the C-seal influences the microbial composition in the days after surgery. 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 bacterial metabolism leading to a reduced production of short chain fatty acids (SCFA) possibly reduces the rate of mucin synthesis by the human host, which in turn may negatively affect wound healing. (3) The C-seal may also create a new ecosystem that benefits the growth of potential opportunistic pathogens. 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, (4) making life hard for commensal oxygen sensitive species while facilitating growth for opportunistic facultative pathogens.

When we analysed the subgroup of non-C-seal patients (standard care), the microbiota of AL versus non-AL was different. The results were similar to the results in **Chapter 2** and showed that a low diversity, with a bacterial composition consisting of mainly (>60%) *Lachnospiraceae* and *Bacteroidaceae,* was correlated with the development of AL. Interestingly, these bacterial families are not particularly known to contain pathogenic bacteria. In fact, many butyrate- producing genera are found within the *Lachnospiraceae* family. Butyrate is a SCFA, known to be the main source of nutrients for colonocytes. (5) Intraluminal injection of SCFAs improved colonic healing, resulted in significantly stronger colonic anastomoses in rats and decreased AL rate. (6,7) Furthermore, butyrate has been shown to regulate the assembly of tight junctions and to correlate with reduced gut permeability. (8) It also decreases intestinal inflammation by reducing oxidative stress in the colonic mucosa. (9)

However, both the abundantly present genera *Blautia* (from the *Lachnospiraceae* family) and *Bacteroides* (from the *Bacteroidaceae* family) are known to comprise species that degrade mucin and produce acetate and propionate or propionate and propanol, but neither of them produces butyrate. (10,11) These bacteria are also associated with the development of inflammatory bowel diseases. (12-14) It could be that these bacteria degrade the mucin-rich mucus-layer around the anastomosis, while a functional mucus-layer is needed for anastomotic healing. (15) Without a mucus-layer the mucus barrier is lost and the inner mucus layer and subsequent colon is vulnerable

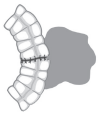
for bacterial penetration. (16) This can cause inflammation and subsequent impaired healing of the anastomosis. (17,18) In addition, a *Bacteroides* dominated microbiome is favoured by protein and animal fat, which can be correlated to the meat consumption as in a western diet. (19,20)

It can also be hypothesized that the lack of bacterial diversity in the AL group the microbiome is less stable than a well-diversified microbiome. The peri-operative aspects in colorectal surgery may cause a larger shift in the bacterial population in a poorly diversified microbiome, offering the opportunity for pathogenic bacteria to thrive. It has been shown that possible pathogenic bacteria like *Enterobacteriaceae*, *Enterococcus*, *Staphylococcus* and *Pseudomonas* are significantly increased after colorectal surgery. (21,22) The administration of prophylactic intravenous antibiotics, starvation and bowel preparation in colorectal surgery all contribute to the increase in amount of such bacteria.

Because the technique (16S analysis) used in **Chapter 2 and 3** is not yet commonly used in clinical practice, other methods to evaluate the microbial diversity could be considered for the prevention of AL. For example, it has been shown that the stool consistency correlates the bacterial richness and diversity (looser consistency means less richness and diversity). (23) This simple observation could be very promising for the risk assessment of AL, but might give a false assumption of the actual diversity of microorganisms at the anastomotic site as faecal samples show a different bacterial composition compared to the composition on and in the mucus layer. (24,25) Therefore our samples, which include the mucus layer give a better representation of the actual situation of the microbial composition at the site of the anastomosis than when we would have used faecal samples.

At the time of performing these studies, the method of 16S analysis was relatively new in the world of surgical research. The methods and statistics used in these studies were subsequently discussed by others after publication. Some of the points that were discussed could indeed be done in a different manner, but in **Chapter 3B** we argued that the 16S analysis is not a "one-fits-all" approach/method. There are multiple ways of preservation, isolation and analysis that can be used. However, the most important factor is that the chosen methods are well-considered and performed in an adequate and consistent way.

We elaborated more on the methodology in microbiome research in **Chapter 4**, where we tried to explain the processes behind uncultured microbial research for the interested surgeon-scientist. This chapter shows that culture-independent microbial studies, such as 16S analysis and metagenomics, in surgery have fallen



behind compared to other specialties in medicine. In our opinion this is not due to the lack of possibilities or research subjects, but mostly due to a lack of knowledge. In order to provide general knowledge on this topic, we wrote a review on the methodology of culture-independent microbial research. In this chapter we show the considerations that have to be made and their pitfalls during the process of 16S analysis.

The 16S gene that is sequenced contains nine hypervariable regions (V1-V9) of which the V4 region is the most widely used, whether or not with additional primers for the V3, V5 and V6 region. The sequence reads should be aligned to one of the 16S reference databases for taxonomic analysis. (26-31) The analysis provides a classification of the sequenced data on the different levels of taxonomic rank (see Table 1).

Table 1 – Example of the scientific classification of bacteria paralleled with the scientific classification of the domestic cat.

Domain/Kingdom	**Bacteria**	**Animalia**
Phylum	Proteobacteria	Chordata
Class	Gammaproteobacteria	Mammalia
Order	Enterobacteriales	Carnivora
Family	*Enterobacteriaceae*	*Felidae* (subfamily Felinae)
Genus	*Escherichia*	*Felis*
Species	*E. coli*	*F. catus (domestic cat)*

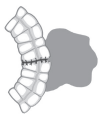
In order to compare the bacterial composition of the samples, diversity analyses are performed. Significant changes in diversity are associated with various diseases. (32-39) The diversity analyses of gut bacterial populations can be conducted within samples (α-diversity) and between samples (β-diversity). (40,41) Numerous methods have been developed in order to create the most accurate representation of the diversity in or between samples, but a golden standard has not been established. Therefore, the best representation of the diversity is dependent on the question of research. It should be noted that the taxonomic, diversity and statistical analysis can be incorrectly interpreted when the data is generated from other mammals than human samples. For example, 85% of the sequences of the mouse microbiome that represent genera have not been detected in humans. (42) Furthermore, the bacterial richness in the mouse intestine seems to be higher compared to that of a human. (42) In addition, every mouse strain has a different microbial composition, which is influenced by their environmental factors like housing, food composition, light, stress factors and pathogen infection. (43) Translation of these data to humans can be challenging.

Although not perfect (yet), the development of next generation sequencing techniques such as 16S, metagenomic and (meta)transcriptomic analyses advances more and more. Such culture-independent analyses can show the presence of the microorganisms and differences in their composition in time. This gives new insights, because the compositional behaviour of microorganisms in their natural habitat differs profoundly from their behaviour in culture. In culture there is a difference in nutrients and resources present and there is neither an interaction between other bacteria nor with the host.

The more advanced (metagenomic and (meta)transcriptomic) analyses make it possible to examine all organisms (e.g. host and microorganisms) present in the sample and simultaneously analyse all the genes or gene expressions of those organisms. This provides not only insight into community biodiversity, but also in the function of the present organisms. Therefore, once metagenomic (and metatranscriptomic) analyses has become easier accessible and more cost-effective, it will improve patient care. However, until both 16S sequencing and metagenomics become more widespread, the cultivation of bacteria will remain the standard of care for patient derived pathogen identification and antibiotic resistance profiling.

In our opinion, the bacterial composition is not the only aspect that plays a role in the development of AL. Although the 16S analysis we used in **Chapter 2 and 3** does show a difference in the microbiota present, we hypothesized that the biological processes within the patient's colon play a role in AL as well. Therefore, we analysed the same samples from **Chapter 2 and 3** with another so-called Next Generation Sequencing technique that has come to rise: RNA-sequencing. We used this technique in **Chapter 5** to identify the gene expression and biological pathways that might be involved in the development of AL. This study presents a transcriptome analysis of the doughnuts from the C-seal trial. It shows that despite normal macroscopic appearance during surgery, there are several differences in gene expression between patients who develop AL and patients who do not. The majority of the differentially expressed genes are downregulated at the moment of surgery in patients who in the end develop AL. The downregulated genes enrich for processes involved in *immune response, angiogenesis, protein synthesis and collagen crosslinking*. These processes can all be related to wound healing, in which roughly three phases are identified: inflammation, proliferation and remodelling.

During the inflammatory phase, there's an influx of immune cells (e.g. neutrophils) in order to prevent infiltration of microorganisms and prevent subsequent infection. The downregulation of genes enriching for the (innate) immune response in an environment with an overabundance of microorganisms could cause an unfavourable



position for the healing colon after surgery and may be an important factor in the development of AL.

The angiogenic process, resulting in adequate supply of oxygen and nutrients, is one of the pillars of the proliferation phase of wound healing. (44) This phase of intestinal healing usually starts after three days after the creation of a wound. (44,45) The healing of colonic anastomoses is considered to be more dependent on angiogenesis (microvasculature) than on diffusion of oxygen through pre-existing macrovasculature. (46,47) Therefore, a downregulation of angiogenesis could contribute to the development of AL.

The remodelling phase of the wound healing process is mainly collagen restructuring. Therefore, the balance of collagen production is an essential part of the healing of the anastomosis. (47-49) The downregulation of collagen crosslinking could cause an inadequate collagen deposition and therefore contribute to AL.

The downregulation of the immune response and collagen crosslinking could also make the patient susceptible for certain microorganisms shown to be involved in AL, such as *E. faecalis* and *P. aeruginosa*. (49,50) For example, *E. faecalis* is known to adhere to wound sites by upregulating its aggregation substance gene (ace) in response to stress situations, like surgery on the host, mechanical bowel preparation or antibiotic treatment. Moreover, *E. faecalis* activates its gelatinase (GelE) gene which degrades collagen and cleaves the pro-form into the activated form of Matrix Metalloprotease 9 (MMP9), an enzyme that degrades extracellular matrix.

Another example of a species that has the "leak phenotype", thus the ability to degrade collagen by GelE and the ability to activate MMP9 is shown in **Chapter 6.** We found that a strain of *Bacillus subtilis* expressed these virulence factors. *B. subtilis* is a commensal mouth organism that is normally not considered to be a pathogen. This strain was cultured from an anastomotic leakage at the site of a gastrojejunostomy after gastrectomy and was tested by the protocol (or workflow) we presented in this chapter. We studied its ability to express a "leak phenotype" based on collagenase production, MMP9 activation and ability to cause a clinical leak in a mouse model (Figure 1). This protocol can be used to determine whether an isolated microorganism has a "leak phenotype" and whether it has the ability to cause anastomotic leakage. As mentioned, mice have a different microbiome and respond differently to bacteria than men, however the *B. subtilis* studied in this chapter is isolated from a human leak.

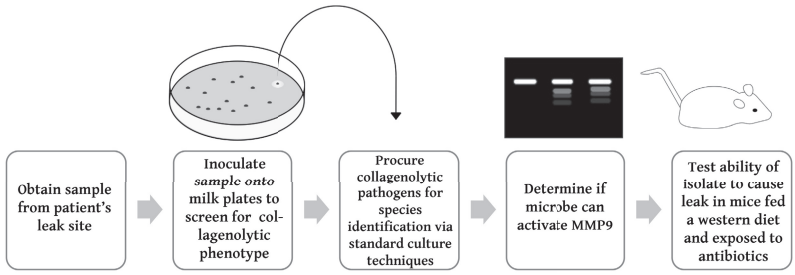
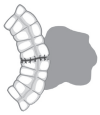


Figure 1 – Workflow to determine the possible contribution of microorganisms in a clinical anastomotic leakage.

The collagenolytic abilities of bacteria have been proven to degrade collagen in rat and mice, but are not tested in human models. (49,50) The effect on human collagen degradation could be questioned, because collagen is degraded at a different rate among mammals. (51) However, mice seem to have a good resemblance with human collagen and extracellular matrix (ECM) and thus mouse models can be considered representative for most ECM and collagen studies. (52,53) Moreover, *B. subtilis* (and other bacteria) has the ability to activate both human and mouse MMP9 and human and mouse MMP9 share orthology. (54) The activation of MMP2, which has similar collagen degrading effects to MMP9, by bacteria and its role in AL has not been studied yet.

The study described in **Chapter 6** suggests a role for *B. subtilis* in the leakage observed. However, it remains to be determined to which extent colonization of a highly collagenolytic bacteria that can also activate MMP9 at the anastomotic site contributes to the clinical manifestation of leakage. It shows that in the current era of promiscuous antibiotic use, the intake of a high fat/low fibre western type diet and the prevalence of obesity, the identification of microbial organisms and the phenotype they express at sites of anastomotic leakage may be important to develop future approaches for intestinal antisepsis prior to gastrointestinal surgery. This is supported by a recent study where mice fed a western diet that undergo surgery (30% hepatectomy) become highly susceptible to lethal sepsis with the administration of antibiotics. (55)

Bacteria that have a "leak phenotype" might play a larger role in colon pathogenesis than just in anastomotic leakage. The microorganism *Enterococcus faecalis,* known to express a leak phenotype, (49) is also associated with colorectal cancer. (56-60) In **Chapter 7** we show that colonization of a collagenolytic strains of *Enterococcus faecalis* and *Proteus mirabilis* in co-incubation with shed cancer cells is associated with extra-intestinal tumour formation. These collagenolytic bacteria were significantly



enriched in the colon of mice fed a high fat, western type diet. This is intriguing given the known role of collagenolytic bacteria in the pathogenesis of an anastomotic leak combined with the known fact that the incidence of colorectal cancer recurrence is associated with both anastomotic leakage and consumption of a high fat, western diet. (49,61,62)

Our attempt to reduce tumour formation by decontaminating mice prior to surgery from collagenolytic bacteria (*E. faecalis* and *P. mirabilis*) using multiple antibiotics failed. Although after decontamination, *E. faecalis* and *P. mirabilis* were not present in the colon, an emergence of highly collagenolytic *Candida parapsilosis* on culture plates was seen. Another attempt to reduce tumour formation was the oral administration of polyethylene glycol (PEG) solution, were phosphate is covalently linked to high-molecular-weight polyethylene glycol (Pi-PEG). It is known that in a phosphate-depleted environment, microbes express enhanced virulence and Pi-PEG has shown to be able to increase the local phosphate concentration and consequently decreases microbial virulence. (50,63-66) Pi-PEG indeed inhibited collagenase production from *E. faecalis, P. mirabilis* and *C. parapsilosis.* Moreover, a reduction in tumour formation was observed. While further confirmatory studies are still needed, we theorize that the efficacy of Pi-PEG to reduce tumour formation lies in its ability to preserve the integrity of the normal microbiota and suppressing commensals from expressing a virulent collagenolytic phenotype.

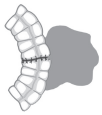
We think that our findings can be used to find targets for the development of therapeutic strategies prior to surgery for the colon at risk for developing AL. Furthermore, they can be used for risk assessment and finally, prevention aimed at modulation of the luminal environment or manipulation of the gut microbiota, might help to reduce the possible pathogenic effects of these inhabitants. An already existing example is the application of selective decontamination at the anastomotic site gives less AL (3.3% vs 7.6% in control group) by eradicating a significant proportion of the bacteria present. (67) However, eradicating bacteria has its down sides since they are also important in the healing of (intestinal) wounds. (68) A better solution might be to prevent bacteria to become virulent. A promising example is the use of Pi-PEG, which reduces the virulence of intraluminal bacteria without changing the microbial composition. (65) However, in this thesis we've shown that a low microbial diversity, possibly caused by a high fat/low fibre western diet, is linked to AL because this might give pathogenic bacteria to thrive. Therefore, we would prefer a prevention that is based on the dietary behaviour of the patient by providing natural sources of nutrients that improve microbial diversity and keep the bacteria from becoming virulent. How, when and for how long the dietary behaviour of the patient should be influenced has

not yet been established. A study by Adriaansens *et al*. showed that a dietary change in 10-12 weeks-old mice from a high fat/low fibre western diet to a low fat/high fibre diet two days before surgery decreases the amount of collagenolytic bacteria present in the expelled stool and reduces AL (data not yet published). Translating two days of prehabilitation in mice to a human equivalent would account for almost one year of life; (69) although it's unknown whether this accounts for the microbial composition as well. In addition, the human gut microbiome shows so called enterotypes which are distinct bacterial compositions. (70,71) The enterotypes are associated with geographical and environmental factors and are age-related, but are not related to the genetic traits of the host. (72,73) Although a small study showed that the microbial stool composition can be changed in one day, the enterotypes are mainly associated with long-term diet and are very hard to change. (19,74,75) Another study shows that a diet can change the microbial composition in faeces significantly in 10 days. (20) The effects of these short-term diets on the microbial composition and their virulence factors in the mucus and thus at the anastomotic site of the human intestine remains unknown.

CONCLUSION

The studies described in this thesis show a new point of view in elucidating the mechanisms behind anastomotic leakage with the investigation of the role of gut microbiota and the intestinal transcriptome. The studies give an indication of the multifactorial aspects involved in intestinal wound healing and thus anastomotic leakage.

Many risk factors for AL had been established thus far, but we show that the mechanisms of developing AL should be sought behind the known risk factors. Our studies show that the intestinal microbial composition was be predictive for the development of AL. When the microbial diversity is low at the time of surgery, it might be easily disturbed by peri-operative interventions. This may cause the colonised microbes to adjust to the new environmental context and become pathogenic. The current approach of intestinal antisepsis with antibiotics prior to gastrointestinal surgery does not consider the microbial phenotype expression as a target. For example, when competing organism are eliminated by broad spectrum antibiotics, bowel preparation or surgery, the remaining organisms can activate their virulence factors and thus shift to a different phenotype by the lack of resistance by other organisms. This could result in colonization of bacteria with a "leak phenotype", causing an impaired healing of the anastomotic wound. Normally the human body is capable to resist these influences of pathogenic bacteria. However, in combination with an altered



gene expression status of the patient's colon at the moment of surgery this might be detrimental for the healing of the anastomosis.

Therefore, a better understanding of the patient's microbiome prior to surgery, the organisms that we eliminate with current antibiotic regimens and the organisms that subsequently colonize in the healing tissues, may be essential for understanding and, with that, future prevention of AL. We believe that our findings show that a one-size-fits-all approach of peri-operative interventions is not ideal.

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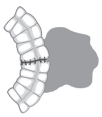

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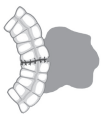

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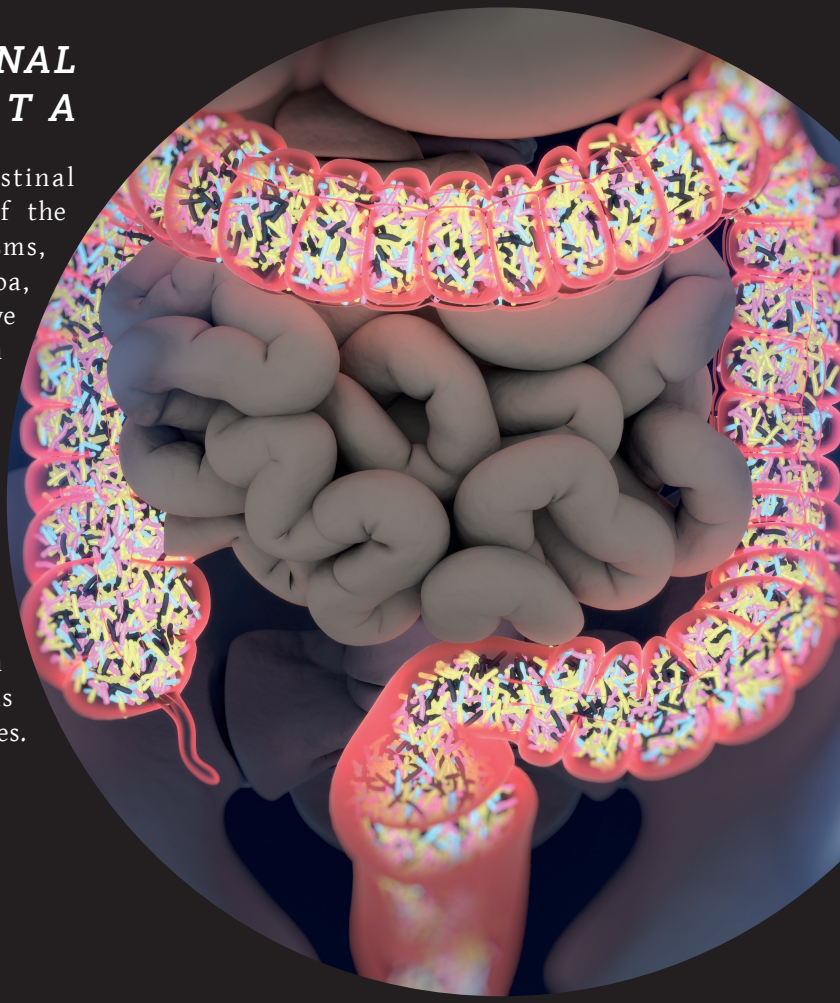
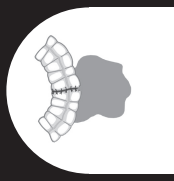
GASTROINTESTINAL MICROBIOTA

The gut or gastrointestinal microbiota is comprised of the totality of microorganisms, bacteria, viruses, protozoa, and fungi, and their collective genetic material present in the gastrointestinal tract. It is considered an important partner of human cells, interacting with virtually all human cells. Gut microbiota dysbiosis, resulting from alterations of composition and function of the gut microbiota and disruption of gut barrier function is associated with many diseases.





CHAPTER 9

DUTCH SUMMARY
NEDERLANDSE SAMENVATTING

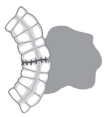
SAMENVATTING VAN DIT PROEFSCHRIFT

Colorectale kanker is een van de meest voorkomende maligniteiten ter wereld, met meer dan een miljoen nieuw gediagnosticeerde patiënten per jaar. De incidentie is het hoogst in de westerse landen, wat voornamelijk is geassocieerd met een westerse leefstijl: onvoldoende fysieke activiteit, te veel (bewerkt) voedsel met veel onverzadigde vetten en suikers en juist weinig vezels. Deze hoge kankerincidentie heeft geleid tot secundaire preventiemaatregelen in de vorm van het bevolkingsonderzoek, waarbij de ontlasting wordt getest en, indien positief, wordt gevolgd door een coloscopie.

De (curatieve) behandeling van colorectale kanker omvat in de meeste gevallen resectie van de tumor met nadien het aanleggen van een anastomose (of ook wel naad genoemd). In vergevorderde ziektestadia wordt dit gecombineerd met neo-adjuvante chemo- en/of radiotherapie. Echter, een gevreesde complicatie van deze operatie is lekkage van de anastomose, oftewel naadlekkage. Deze complicatie komt bij ongeveer 10% van de operaties voor en leidt tot veel morbiditeit, interventies, verlengde ziekenhuisopnames, hoge kosten en verhoogde mortaliteit. Tevens wordt er bij naadlekkage een grotere kans op lokale recidief tumorvorming gezien en daardoor een verminderde ziektevrije overleving.

Ondanks steeds geavanceerdere technieken en het in kaart brengen van risicofactoren, zoals co-morbiditeiten (bijv. Diabetes Mellitus), grotere tumoromvang en het gebruik van medicamenten als corticosteroïden, is de incidentie van naadlekkage nauwelijks gedaald in de afgelopen jaren. Deels is dit het resultaat van de nog altijd beperkte kennis over het ontstaansmechanisme van naadlekkage. Hoewel naadlekkage wordt gezien als falende of abnormale wondheling, is er nog weinig bekend over wondheling in het gastro-intestinale traject. Tot nu toe heeft men aangenomen dat de wondhelingsprocessen in de darm grotendeels hetzelfde zijn als elders in het lichaam.

In **hoofdstukken 2 en 3**, worden studies beschreven waarbij met 16S rRNA gene sequencing de bacteriële samenstelling is geanalyseerd van monsters verzameld tijdens operaties waarbij een colorectale anastomose werd aangelegd. De monsters zijn de “donuts” die bij het staplen (nieten) van een anastomose losgesneden worden op de plek van de anastomose om de continuïteit van de darm te herstellen. De patiënten waren geïncludeerd in de C-seal trial, waarna zij vervolgens gerandomiseerd werden tussen de operatiegroep waarbij een “normale” anastomose werd aangelegd (standaardprocedure) of de groep waarbij de anastomose met een C-seal werd verricht (onderzoeksprocedure). De C-seal is een biologisch degradeerbare intraluminale sheet, ontworpen om naadlekkage te voorkomen. In **hoofdstuk 2** wordt een pilot-studie



beschreven waarbij alleen monsters van 16 patiënten (8 met naadlekkage en 8 zonder naadlekkage) zonder C-seal zijn onderzocht.

Ondanks dat de body mass index (BMI) van patiënten die naadlekkage ontwikkelden iets hoger was, was dit geen significante of onafhankelijke factor voor het ontstaan van naadlekkage bij deze patiënten. Er werd tevens geen significant verschil gevonden in de aanwezige bacteriën tussen patiënten met of zonder lekkage. Echter, de patiënten die naadlekkage ontwikkelden bleken een significant lagere microbiële diversiteit te hebben. Deze lage diversiteit werd gekenmerkt door een hoger percentage van bacteriën uit de bacteriële families *Lachnospiraceae* en *Bacteroidaceae.*

In **hoofdstuk 3** is een groter cohort onderzocht, waarbij ook monsters van patiënten die een C-seal kregen zijn geanalyseerd. Deze analyse van 118 monsters liet, tussen patiënten die naadlekkage ontwikkelden en normaal helende naden geen verschillen in bacteriële compositie zien, op een overvloed aan de *Blautia* genus in naadlekkagepatiënten na. Het ontbreken van verschillen in bacteriële compositie in de totale groep kan met name worden toegeschreven aan de patiënten met de C-seal. De microbiële compositie van deze monsters verschillen niet significant, maar in de C-seal trial werd er juist een trend gezien naar meer naadlekkage in C-seal patiënten dan bij patiënten zonder C-seal. Dit suggereert dat de C-seal de microbiële compositie verandert in de dagen na de operatie, mogelijk doordat het een barrière creëert tussen de mucosa en de (verse) luminale inhoud.

De analyse van de subgroep van niet-C-seal patiënten (standaardoperatie) liet zien dat de microbiota van naadlekkagepatiënten verschilde met die van controlepatiënten. De resultaten kwamen overeen met de resultaten van **hoofdstuk 2**. Ook in dit grotere cohort hebben patiënten met naadlekkage een lagere diversiteit in microbiële compositie, voornamelijk bestaand uit een overvloed aan de bacteriële families *Lachnospiraceae* en *Bacteroidaceae* (>60%). Interessant genoeg staan deze families niet bekend om hun pathogene bacteriën. Echter, mogelijk door het gebrek aan diversiteit is de balans binnen microbiële compositie minder stabiel dan de meer gevarieerde microbiota samenstellingen. Hierdoor zou deze balans door perioperatieve interventies eenvoudiger kunnen “omvallen” en vervolgens de kans bieden aan pathogene bacteriën om de overhand te krijgen.

Tevens is er bij naadlekkagepatiënten een significante overvloed van de genera *Blautia* (van de *Lachnospiraceae* familie) en *Bacteroides* (van de *Bacteroidaceae* familie). Deze genera bevatten bacteriële species (soorten) die mucine degraderen. Het zou kunnen dat deze bacteriën de mucinerijke mucuslaag rond de anastomose, die essentieel is voor de heling van de anastomose, afbreken. Zonder mucuslaag is de fysieke barrière

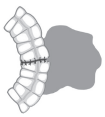
die het vormt verdwenen en is het colon vatbaarder voor bacteriële penetratie, wat weer kan leiden tot inflammatie en verminderde heling van de anastomose.

De gebruikte 16S analyse is een relatief nieuwe methode in het chirurgisch onderzoek. De methodes en statistieken van deze studies werden bediscussieerd door anderen, waarop in **hoofdstuk 3B** wordt ingegaan. In dit hoofdstuk wordt beargumenteerd dat de 16S analyse (nog) geen "gouden standaard" heeft en de analyse afhankelijk is van vele factoren.

In **hoofdstuk 4** wordt verder ingegaan op de methodologie achter microbioomonderzoek. In de chirurgische onderzoekswereld wordt nog steeds weinig gebruik gemaakt van deze methodologieën. Omdat er nog weinig kennis over is worden de kweekonafhankelijke microbiële studies, zoals de 16S analyse en metagenoomstudies, in detail besproken.

Onze hypothese is dat niet alleen de bacteriële compositie een rol speelt in de ontwikkeling van naadlekkage, maar dat de biologische processen in het gastro-intestinale weefsel tevens van invloed zijn. Daarom hebben we dezelfde monsters van hoofdstuk 2 en 3 gebruikt voor een andere techniek: RNA-sequencing van het weefsel. In **Hoofdstuk 5** wordt deze techniek gebruikt om de genexpressie en biologische processen te onderzoeken die betrokken zouden kunnen zijn bij de ontwikkeling van naadlekkage in de donuts die verzameld zijn tijdens de C-seal trial. Het verschil in genexpressie is voornamelijk een grote hoeveelheid down-gereguleerde genen in patiënten met naadlekkage ten opzichte van patiënten met normale heling. Middels een co-functionaliteitanalyse blijken deze down-gereguleerde genen voornamelijk te verrijken voor processen die betrokken zijn bij o.a. een verminderde expressie van genen betrokken bij de immuunrespons, bloedvatnieuwvorming en collageenopbouw. Deze processen kunnen allemaal worden gerelateerd aan wondheling.

In **hoofdstuk 6** wordt een bacterie beschreven die het "naadlekkage fenotype" heeft. De commensale mondbacterie *Bacillus subtilis,* die over het algemeen niet als pathogeen wordt bestempeld, blijkt de virulentiefactoren te bevatten die dit fenotype bepalen. Een ras (strain) van *B. subtilis* was gekweekt uit lekkagevocht van een patiënt met naadlekkage van een gastrojejunostomie. Dit isolaat werd volgens het protocol beschreven in **hoofdstuk 6** geanalyseerd op de virulentiefactoren: 1) de eigenschap om collagenase te produceren; 2) om MMP9 te activeren; en 3) om een klinische naadlekkage te veroorzaken in een muismodel. Dit protocol kan gebruikt worden om een geïsoleerd micro-organisme te testen op een "naadlekkage fenotype".



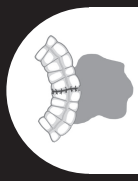
Bacteriën met een "naadlekkage fenotype" spelen misschien zelfs een grotere rol bij de colonpathogenese dan alleen bij naadlekkage. Het micro-organisme *Enterococcus faecalis,* waarvan bekend is dat het "naadlekkage fenotype" kan hebben, is ook geassocieerd met colorectale kanker. In **hoofdstuk 7** blijkt dat de kolonisatie van collagenalytische *E. faecalis* en *Proteus mirabilis* in muizen met een colonoperatie wordt geassocieerd met extra-intestinale tumoren. Deze collagenalytische bacteriën waren met name significant verhoogd in het colon van muizen met een vetrijk/vezelarm westers dieet.

Een poging om in dit model de tumorformatie te reduceren door de muizen te ontdoen van collagenalytische bacteriën (*E. faecalis* en *P. mirabilis*) met meerdere antibiotica voorafgaand aan de operatie was echter niet succesvol. Ondanks dat na de decontaminatie *E. faecalis* en *P. mirabilis* niet meer in het colon aanwezig waren, was er groei van een andere hoog-collagenalytische species (*Candida parapsilosis).* Een andere poging om de tumorformatie te reduceren was middels de orale toediening van een gefosforesceerde vorm van polyethyleen glycol (PEG), genaamd Pi-PEG. Het is namelijk bekend dat in een fosfaatarme omgeving, microben een verhoogde virulentie tonen. Pi-PEG kan de lokale fosfaatconcentratie verhogen en de microbiële virulentie verlagen. Dit vonden wij ook in de studie in **hoofdstuk 7**, waar het de collageenproductie van *E. faecalis*, *P. mirabilis en C. parapsilosis* verminderde. Tevens werd een reductie van tumorformatie gezien. Meer studies zullen nodig zijn om dit te bevestigen, maar de hypothese is dat de effectiviteit van Pi-PEG om tumorformatie te reduceren in het behouden van de integriteit van de normale microbiota ligt en daarmee de virulentie van commensalen kan onderdrukken.

Wij denken dat onze bevindingen in dit proefschrift kunnen worden gebruikt om toekomstige therapeutische strategieën te ontwikkelen om naadlekkage te voorkomen. Tevens kunnen onze bevindingen worden gebruikt voor het inschatten van het risico dat patiënten lopen op postoperatieve naadlekkage. Tot slot denken wij dat preventiemaatregelen gericht op het interne milieu van de darm en/of manipulatie van de intestinale microbiota kunnen helpen bij het reduceren van mogelijke pathogene effecten van deze bewoners van de darm.

A P P E N D I X

The appendix is a small, pouch-like sac of tissue that is located in the first part of the colon (cecum) in the lower-right abdomen. Lymphatic tissue in the appendix aids in immune function, but simultaneously harbors bacteria. The official name of the appendix is veriform appendix, which means “worm-like appendage.” Of course, it also just means a section or table of subsidiary matter at the end of a book or document.



APPENDICES

DANKWOORD
PUBLICATIES
OVER DE AUTEUR

DANKWOORD

Dit proefschrift is uiteindelijk een product dat voortgekomen is uit de tijd die velen in mij hebben gestoken. In alle vormen van tijd en alle vormen van contact; van correcties tot discussies en van kritieken tot liefde. Daar ben ik dan ook ongehoord dankbaar voor. Ik ontkom er dan ook niet aan om de belangrijksten uit te lichten en mijn dank te uiten.

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Beste Klaas, zonder jouw oprechte wil om naadlekkage te verminderen en de ellende die het met zich meebrengt te reduceren was dit boekje niet zoals het nu is. Wij ontmoetten elkaar toen ik bij het C-sealteam mocht komen. Gedurende de trial nam ik steeds meer op me en hadden wij frequenter contact. Daardoor was de stap naar een leuk artikeltje makkelijk gemaakt. Vervolgens kwam mede door jouw enthousiasme en out-of-the-box denken een climax dat we samen tot een proefschrift hebben gemaakt. Bedankt ook voor al je momenten van eindeloos sparren, discussiëren en natuurlijk voor de mooie tripjes naar onder andere de boot, (bijna) alle ziekenhuizen in Nederland, Nashville en Chicago.

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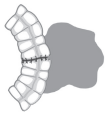
Graag zou ik ook mijn leescommissie willen danken voor de aandacht en kritische beoordeling van dit proefschrift. Professoren R.K. Weersma, P.J. Tanis en H.G.D. Leuvenink, bedankt voor jullie evaluatie van dit proefschrift. Daarbij spreek ik mijn



speciale dank uit naar Henri, vanwege zijn betrokkenheid bij mijn allereerste (totaal niet aan dit proefschrift gerelateerde) onderzoekje, en dat zijn deur altijd voor mij openstond om het over vele uiteenlopende ideeën te hebben.

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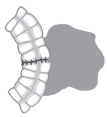

bedankt ook voor alle *lunchos* samen. De vele vrijdagmiddagborrels in Block & Barrels waren frequent nodig, maar zijn vooral gewoon heel erg gezellig. Guido, I know you can read the Dutch part, but since I'm still not used to talking Dutch to you: Thanks for being my friend and for hearing about my shit, for sharing your shit and for thinking together about shit in all forms.

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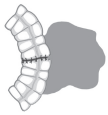

hebben gesteund in al mijn worstelingen. Bedankt ook dat ik af en toe mijn onderzoek urenlang tot in de kleinste details uitgebreid mocht uitleggen in een voor jullie zo duidelijk mogelijke taal en bedankt dat jullie het volhielden te luisteren zonder in slaap te vallen.

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Intestinal Microbiota and Anastomotic Leakage of Stapled Colorectal Anastomoses: A Pilot Study.
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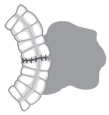

Western Diet Promotes Intestinal Colonization by Collagenolytic Microbes and Promotes Tumor Formation Following Colorectal Surgery.
Sara Gaines, Jasper B. van Praagh, Ashley J. Williamson, Richard A. Jacobson, Sanjiv Hyoju, Alexander Zaborin, Jun Mao, Hyun Y. Koo, Lindsay Alpert, Marc Bissonnette, Ralph Weichselbaum, Jack Gilbert, Eugene Chang, Neil Hyman, Olga Zaborina †, Benjamin D. Shogan †, John C. Alverdy †.
Gastroenterology. 2019 Oct 23. [Epub ahead of print]

Infliximab Does Not Promote the Presence of Collagenolytic Bacteria in a Mouse Model of Colorectal Anastomosis.
Sara Gaines, Sanjiv Hyoju, Ashley J. Williamson, Jasper B. van Praagh, Olga Zaborina, David T. Rubin, John C. Alverdy, Benjamin D. Shogan, Neil Hyman.
J Gastrointest Surg. 2020 Jan 2. [Epub ahead of print].

OVER DE AUTEUR

Jasper van Praagh werd op 6 maart 1991 geboren te Amsterdam, Nederland. Hij heeft zijn eerste levensjaren daar doorgebracht, waarna hij het grootste deel van zijn jeugd volbracht in Apeldoorn. In 2009 behaalde hij zijn diploma aan het Gymnasium Apeldoorn, waarna hij in hetzelfde jaar startte met de studie Geneeskunde aan de Rijksuniversiteit Groningen.

Tijdens zijn studietijd zette hij voort waar hij tijdens zijn middelbare school al mee begonnen was: het deelnemen in commissies en het organiseren van evenementen. Daarnaast had hij gedurende de eerste jaren van zijn studententijd enkele bijbaantjes, waaronder zijn werk als clinical research coordinator bij de C-sealtrial. Gedurende deze werkzaamheden ontwikkelde Jasper, onder zijn huidige co-promotor Klaas Havenga, de interesse voor de Chirurgie en de Abdominale Chirurgie in het bijzonder. Nog voor het beëindigen van de bachelorfase van de studie werd er gewerkt aan een wetenschappelijk artikel.

Dit zorgde ervoor dat Jaspers interesse in de wetenschap verder werd gewekt, waarna hij startte met een onderzoek in het kader van zijn Stage Wetenschap op monsters verzameld tijdens de C-sealtrial. Dit leidde tot een eerste publicatie, waarna een MD/PhD-traject aan de Rijksuniversiteit Groningen en het Universitair Medisch Centrum Groningen volgde. Gelijktijdig met de co-schappen werd het onderzoek voortgezet. Dit ging gepaard met verschillende verblijven in het buitenland met in het eerste masterjaar een uitstap naar het Wellcome Trust Sanger Institute te Cambridge in het Verenigd Koninkrijk.

Tijdens het tweede master jaar liep Jasper zijn coschappen in Zwolle, waarna hij voor zijn MD/PhD-traject een aantal maanden bij vooraanstaand onderzoeker en chirurg prof. dr. John C. Alverdy bij de University of Chicago verbleef. Zijn semi-artsstage van de Geneeskundestudie volbracht hij bij de Abdominale Chirurgie in het Universitair Medisch Centrum Groningen, waarna Jasper nog een aantal maanden terugging naar Chicago om verder onderzoek te doen in het Alverdy laboratorium. Gedurende zijn masterfase heeft hij tevens samen met anderen de Vereniging Chirurgie voor Medisch Studenten opgericht, eerst in Groningen om vervolgens het landelijke, overkoepelende orgaan vorm te geven.

Tot slot heeft Jasper ten tijde van zijn promotieonderzoek verschillende (inter-) nationale congressen bezocht, waar hij zijn studieresultaten heeft mogen presenteren. In oktober 2019 startte Jasper met zijn eerste klinische baan als ANIOS bij de Chirurgie in het Deventer Ziekenhuis.