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DISTINCT MICROBIOTA IN PATIENTS DIAGNOSED WITH COLORECTAL CANCER COMPARED TO HEALTHY INDIVIDUALS

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Abstract – *Objective:* The gastrointestinal tract consists of a complex microenvironment with an abundance of microorganisms, and a diverse microbiome composition is evident along the gastrointestinal tract with a favorable growth environment for bacteria. Dysbiosis to this environment have been associated with colorectal cancer (CRC) as well as other diseases, with studies identifying a higher number of bacteria, such as the *Enterobacteriaceae* family: *Clostridium* spp., *Fusobacterium* spp., and *Streptococcus bovis*. We hypothesize that patients with CRC have a distinct microbiota with a greater abundance of atypical *Campylobacter* spp. and *Helicobacter* spp. compared to control patients.

Patients and Methods: Sixty biopsies were collected from forty randomly selected patients in Kaunas, Lithuania. The patients were allocated into groups: patients with CRC and control patients without intestinal cancer or cancer risk factors. Twenty biopsies were collected from each collection site: The tumor and the adjacent intestinal tissue in CRC patients, and a randomly selected intestinal tissue site in non-cancer patients. A microbiome analysis was performed using 16S rDNA and 18S rDNA.

Results: Distinct bacterial microbiota was found to be significantly different in the collected biopsies between CRC patients and control patients with p=.001. There was a clear overrepresentation of *Campylobacter* spp. with *C. rectus* being the most prominent in CRC patients. Moreover, *Fusobacterium* spp. was found in each group, with increased abundance of *F. nucleatum* and *F. necrophorium* in cancer tissue. There was no significant abundance of the *Helicobacter* spp. found.

Conclusions: This pilot study illustrates a significant difference in the gut microbiota in patients with CRC compared to non-cancer patients. The microenvironmental composition in CRC tissue samples were dominated by *Campylobacter* spp. and *Fusobacterium* spp. suggesting that these species either solely or in co-aggregation may cause or promote CRC.

Keywords: Colorectal cancer, Microbiome, Dysbiosis, *Helicobacter* spp, *Campylobacter* spp, *Atypical Campylobacter* spp, *Fusobacterium* spp.

INTRODUCTION

Colorectal Cancer

Colorectal Cancer (CRC) is the third most frequent type of cancer worldwide[1,2]. Although CRC has shown a significant decrease in incidents and mortality over the last decade[2-4], it is still one of the leading causes of cancer. Evidence suggests that cancer symptom unawareness and late diagnosis may explain the high CRC frequency[3,5], and highlights the importance of further research to promote early diagnosis.

The incidences of CRC vary greatly throughout the world. However, research has shown a higher incidence in developed regions, such as North America, Northern and Western Europe compared to developing regions, i.e., Africa. Furthermore, the data presents evidence that people above the age of 50 have a greater risk for CRC, with men having a moderately increased risk compared to women[2].

The development of CRC has been identified in various areas of the colorectal tract. Moreover, a higher frequency was found in the right side of the colon with approximately 30%, hereafter 20% located in the sigmoid colon, 20% in the rectum, 15 % in the left descending colon and finally 10% in the transverse colon[3]. The primary cancers found in these areas are predominantly adenocarcinomas (95%), yet carcinoid tumors, gastrointestinal stromal tumors, lymphomas and polyposis were identified as well[3,6,7]. The biological significance of these locations is yet unknown, however it is widely believed that the majority of CRC is developed by the adenoma carcinoma sequence process[8,9]. This process provides a pathological model for colorectal tumorigenesis, yet it does not consider the microbial pathophysiology or the environmental factors, i.e., body mass, diet or lifestyle[10].

CRC is composed of a complex association of cells including tumoral cells, non-neoplastic cells and a multitude of microorganisms[11,12]. The microorganisms are believed to be one of the major factors in the development of CRC[13-16], and Mizutani et al[13] recently presented evidence that suggests an association between gut microbiota and CRC development. Furthermore, a plethora of studies have suggested that CRC patients presents with dysbiosis, which is linked to the development of CRC[17,18], although further research is needed to characterize the composition of this microbiota and its role in carcinogenesis[13-17].

The Intestinal Microbiota

The human gastrointestinal (GI) tract is the habitat for a complex and dynamic assembly of microorganisms consisting of bacteria, bacteriophage, fungi, protozoa and viruses that is called the human gut microbiota[11,17,19]. The gut microbiota show great influence on gut integrity as well as human health and disease[19]. Dysbiosis to this environment is associated with several GI diseases such as inflammatory bowel disease (IBD) and GI cancers[13,20]. Moreover, studies have shown that environmental factors such as infection, diet and lifestyle form our gut microbiota and metabolism and is believed to impact cancer development[11,13,16,21-23].

Colon constitutes a strongly dense section of microbiota of the GI tract and include approximately 70% of the total microbiota[14]. There are suggestions that some of the bacterial species of the colon may create a microenvironment that is favorable for the pathogenesis of CRC[14]. In example, Marchesi et al[24] found a microenvironment composed of increased lactate, significantly decreased glucose, as well as amino acids, fatty acids and lipids which could indicate an individual variability within the CRC tumor microenvironment. Furthermore, studies suggest a variety of mechanistic evidence for bacterial involvement in the development of CRC, which includes DNA damaging genotoxins, DNA damaging superoxide radicals and induction of cell proliferation by T-cells. Evidence show that they might accelerate the cancer development by manipulating cell metabolism and the immune system although it is not fully understood to this date[24-27].

Furthermore, Winter et al[28] and Flanagan et al[29] theorize that dysbiosis creates a harmful inflammatory response, causing destruction of the intestinal barrier and therefore damage to the bacterial translocation and cytokine secretion, sustaining the inflammatory environ-

ment needed in carcinogenesis. However, it is still unknown whether it is the consequence or the cause of CRC[11]. Nevertheless, several bacteria have been identified and linked to CRC carcinogenesis; *Streptococcus bovis*, *Clostridium septicum, and Fusobacterium* spp., as they have been found in higher numbers in CRC patients compared with control patients[10,11,30]. Hence, these species may have pro-carcinogenic properties and hypothetically other unknown species even though there is no consensus at this point.

Streptococcus bovis

Streptococcus bovis (sb) has been recognized as a malignant factor and been associated with CRC since the early 1950s. Deng et al[31] examined *S. bovis* potential molecular mechanism in CRC and found a higher quantity in CRC patients as well as more advanced TNM. Furthermore, they found that *S. bovis* aggravated tumorigenesis as well as a possible increased recruitment of TLR-4+ CD11b+ cells. Moreover, Saus et al[14] found that overexpression of COX2 *in vitro* promoted cell proliferation and increased number of inflammatory cytokines such as TNF-α, IL-6 and IL-8.

Clostridium septicum

Clostridium septicum infections have variable clinical presentations and a strong association with malignancy as well as high mortality, and *C. septicum* has been found to be associated with CRC[32]. Even though *C. septicum* cases are rare, identification and treatment are of essence due to its malignancy and mortality. Mirza et al[33] argue that *C. septicum* malignancy may be that tumors provides an acidic and hypoxic environment that may provide a conducive spore germination, and therefore lead to infection and colorectal malignancy.

Fusobacterium spp.

Fusobacterium spp. are immobile gram-negative anaerobic rods with variable morphology and size. Their morphology can be described as filamentous or fusiform[34]. *Fusobacterium* spp. constitute as a part of the intestinal flora which participates in the digestion and protects against colonization of pathogenic bacteria. However, bacteria from the intestinal flora, including *Fusobacterium* spp, can also cause infections such as acute appendicitis, bacteremia, colon cancer, IBD, Lemierre's syndrome, and tonsillitis[35].

F. nucleatum is associated with the development of CRC by different mechanisms[26,29]. Several studies[30,36] have identified how higher levels of *F. nucleatum* cause specific molecular tumor incidents, and may induce the microsatellite instability pathway, which is one possible pathway for the pathogenesis of colorectal cancer[37]. Rubinstein et al[26] have depicted how *F. nucleatum* promote CRC tumor cell growth by stimulating the activation β-catenin signaling and described how *F. nucleatum* induce oncogenic gene expression via the FadA adhesion virulence factor[38]. Similar carcinogenic mechanisms of *F. nucleatum* have been identified, which emphasize how it contributes to the development of CRC[39]. *Fusobacterium* spp. demonstrate carcinogenic characteristics and continuous research show association with the development of CRC.

Campylobacter spp.

The genus Campylobacter is a diverse group of bacteria consisting of twenty-six species, two provisional species and nine subspecies[40]. They are gram negative and vary in shape and form; from rod-shaped, curved, or spiral shaped. Depending on the species, they may have polar, bipolar flagella or non-flagellum. The genus requires a complex environment and grow under specific microaerobic and anaerobic conditions[41]. *Campylobacter* spp. are found mainly in the GI tract in addition to the oral cavity in humans, and are known to cause diseases such as ga-

stroenteritis, IBD, ulcerative colitis, Chron's disease, CRC as well as periodontal abscesses. They are also reported in extra gastrointestinal manifestations such as bacteremia, septicemia, and brain abscesses[40,42]. The *Campylobacter* spp. predominantly known to cause gastrointestinal diseases are *C. jejuni* and *C. coli*, although in recent years a wide range of species in the genus have been identified: *C. helveticus*, *C. showae*, *C. fetus*, *C. hyointestiucus*, *C. rectus* and *C. concisus*[40,42,43]. Furthermore, *C. gracilis* is found in periodontal diseases and IBD, similar to *C. ureolyticus* which is associated to periodontal diseases as well as gastroenteritis and IBD[40]. The precise pathogenesis of how these *Campylobacter* spp. interact with the variety of diseases are not well known. Tjalsma et al[44] suggest a driver- passenger model in CRC. The model proposes that the so-called "driver bacteria" are certain bacteria from the intestine, such as Enterobacteriaceae, that initiate epithelial DNA damage and promote inflammation. Moreover, the "passenger bacteria" in particular, *Fusobacterium* spp. take advantage of the newly initiated tumorigenesis and proliferated environment as passage into the new environment. Tjalsma et al[44] highlight that passenger bacteria are the dominant bacteria that thrive in CRC, however they specify that it is still unknown whether they promote or suppress tumor development. Conversely, Singh et al[45] strongly suggest that chemokines are the key mediators between tumor cells and their microenvironment, and overexpression of chemokines are the hallmark of tumor progression. Ning and Lenz[46] further support this theory by suggesting chemokines and their receptors are upregulated in CRC, especially IL-8 and its receptor CXCR2. It is possible that CRC may be promoted by multifactorial signals and mechanisms, yet further studies are needed to specify the precise pathogenesis.

Helicobacter spp.

Helicobacter spp. is a gram-negative genus that includes more than 35 species with the *H. pylori* being the most well-known[34]. There are a growing number of non-*Helicobacter pylori* helicobacters (NHPH) that increases our knowledge of the multitude of *Helicobacter* spp. and how the bacteria colonize, not only the human gastrointestinal tract but the body in general[47].

Of the gastric helicobacters, *H. pylori* is known to be the dominant gastric pathogen causing gastroduodenal ulceration, gastric cancer (primarily adenocarcinomas), gastritis, Mucosa-associated lymphoid tissue (MALT) and lymphomas in humans[4,48]. However, recent studies have shown that other gastric helicobacters like *H. felis*, *H. heilmannii*, *H. salomonis* and *H. suis* are associated with gastroduodenal inflammation, ulcus disease and gastric cancers as well[49]. Peng et al[38] suggested that there might be similar traits in the metabolism and chemtaxic genes between *H. pylori* and the gastric helicobacters, enabling the different bacteria to survive and colonize in different gastrointestinal environments. Interestingly, they found that *H. suis* and *H. felis* had a wider metabolic flexibility than *H. pylori* and therefore a broader response to environment signals. Moreover, *H. pylori, H. felis, H. salomonis* and *H. suis* displayed significant differences in their genomes and pathogenicity. Nevertheless, *H. pylori*, *H. felis*, *H. salomonis* and *H. suis* demonstrate similar properties by decreasing acid secretion and utilize urease activity, which can enhance the associated surrounding growth[38].

The major difference between intestinal and gastric *Helicobacter* spp. is that most intestinal *Helicobacter* spp. lack the urease activity[50], although the urease activity of *H. pylori* has not been described as a carcinogenic factor. The World Health Organization (WHO) has classified *H. pylori* as a class 1 carcinogen, even though the precise mechanism by which *H. pylori* cause cancer is not known[51]. Therefore, it might be assumed that the carcinogenic genes in gastric *H. pylori* also could be present in some of the intestinal *Helicobacter* spp. which may affect development of CRC.

MATERIALS AND METHODS

Patients and Sampling of Intestinal Biopsies

This pilot study included a total of forty randomly selected patients, all recruited from Kaunas, Lithuania. Patient consent was collected and verified by the department of ga-

stroenterology, Kaunas, Lithuania. The patient groups were divided into the following groups: Patients with CRC, and patients without CRC or risk factors for cancer. A total of sixty biopsies were collected. Twenty biopsies were extracted from the tumor site (cancer tissue), another twenty biopsies were extracted from the adjacent normal intestinal tissue (adjacent tissue) of cancer patients. The last twenty samples were from a random intestinal tissue site in non-cancer patients (control tissue). All samples were marked anonymously, and the identity of the subjects were not known.

The biopsies were stored at -80°C until all sixty samples were collected and then shipped on dry ice to the Department of Clinical Microbiology, Rigshospitalet, Copenhagen.

ANALYSIS/EXAMINATION OF BIOPSIES

Microbiota Analysis

The biopsies were brought on dry ice to Statens Serum Institut where DNA was extracted from the biopsies, and the microbiome was analyzed by 16S rDNA analysis and 18S rDNA analysis.

DNA Extraction

The DNA extraction of the intestinal biopsies was performed with a QIAamp DNA mini-Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction for tissues. A negative control with no material from samples was included for downstream analysis for each batch of DNA extraction.

Primer design

The extracted DNA was amplified by a two-step PCR using primer sets targeting the 16S rDNA gene and 18S rDNA.

Library Preparation and Sequencing

The purified 16S rDNA were initially amplified by the same procedure as in the previous publication by Spiegelhauer et al[52]. The purified 18rDNA were initially amplified by the same procedure as in the previous publication by Hansen et al[53].

Bioinformatics

We used BION-META (http://box.com/bion) for analyzing the sequence data from the 16S rDNA and 18S rDNA gene sequencing. It allows non-overlapping paired reads for analysis and is often accurate to the species level.

STATISTICAL ANALYSIS

Analysis of microbiota composition was performed in R version 3.5.0 using the packages phyloseq v. 1.24.2 and vegan v. 2.5-2. Figures were created using ggplot2 v. 3.2.0. Alpha diversity of samples as well as relative abundances of individual genera were compared between groups with Wilcoxon rank sum tests and adjusted for multiple testing using Bonferroni correction.

RESULTS

Distinct Bacterial Microbiota Observed Between CRC Patients and Patients Without Cancer

In Figure 1 it is observed that the CRC patients have a distinct bacterial microbiota compared to control patients without intestinal cancer or cancer risk factors. This is confirmed by a ANOSIM test, which demonstrates that the bacterial microbiota in gastric biopsies from CRC patients and patients without cancer are significantly different (p=.001).

Abundance of *Campylobacter* spp. and *Fusobacterium* spp.

In Figure 2 we observe the abundance of *Campylobacter* spp. and *Fusobacterium* spp. In relation to the sample site. It is evident that there is no significant difference in the abundance of *Fusobacterium* spp. between the tissue samples from patients without cancer, adjacent tissue, and the cancer tissue with p=.05. However, the abundance of *Campylobacter* spp. shows a substantial increase from the tissue samples from patients without cancer, to the cancer tissue with a significant p-value of p=.029. There is a clear overrepresentation in the cancer tissue.

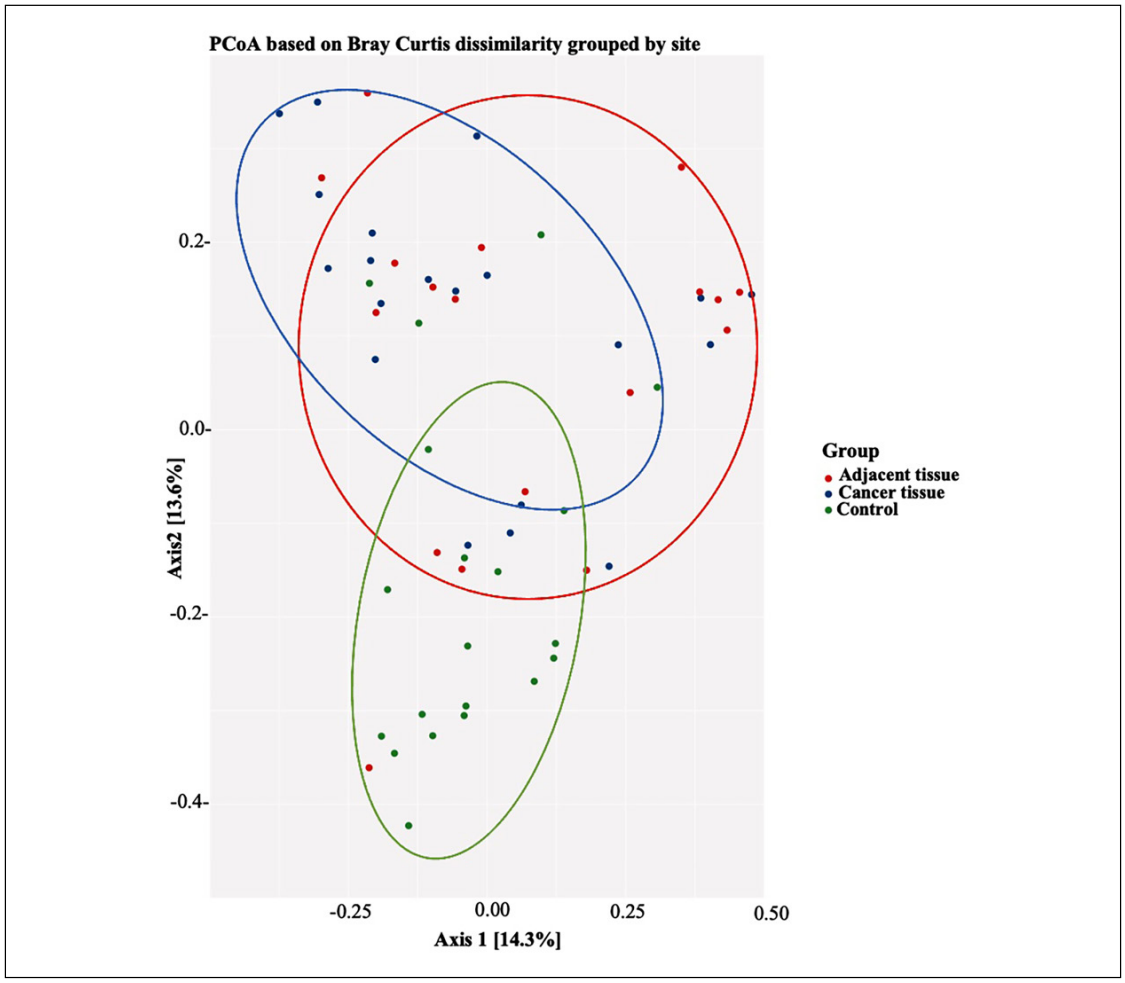

Figure 1. Beta diversity visualized by Principal Coordinates Analysis (PCoA) using Bray Curtis dissimilarity. Each dot on the plot represents the total bacterial community of one sample. The distances between the dots represent the comparable microbial community between samples. Samples are colored by three sampling groups. The colors red (adjacent tissue) and blue (cancer tissue) represent samples from cancer patients and the color green represents samples from control patients. PERMANOVA test p = .001.

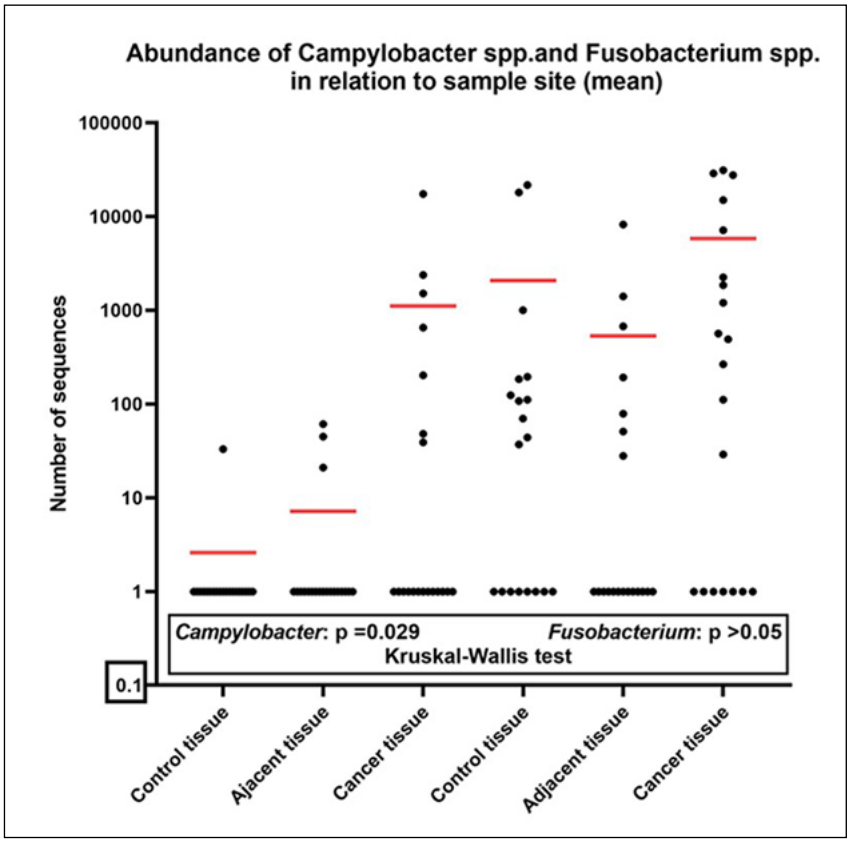

Figure 2. Scatterplot showing the abundance of *Campylobacter spp.* and *Fusobacterium spp.* Kruskal Wallis test on control tissue and cancer tissue; Campylobacter (p = .029), *Fusobacterium* (p > .05). Figure 2 illustrates the abundance of *Campylobacter spp.* and Fusobacterium spp. in three different sample sites: patients without cancer or cancer risk factors (control tissue), cancer tissue from patients with colorectal cancer and tissue without cancer from patients with colorectal cancer (adjacent tissue).

Abundance of *Campylobacter* spp.

Figure 3 shows the different *Campylobacter* spp. represented. This includes *C. concisus*, *C. gracilis*, *C. rectus*, *C. showae* and *C. ureolyticus*. The Figure 4 shows an increase of *Campylobacter* spp. in the cancer tissue compared to the adjacent tissue and the control tissue where the abundance is less distributed. In the cancer tissue it is clear that *C. rectus*, *C. ureolyticus* and *C. showae* which have the highest abundance.

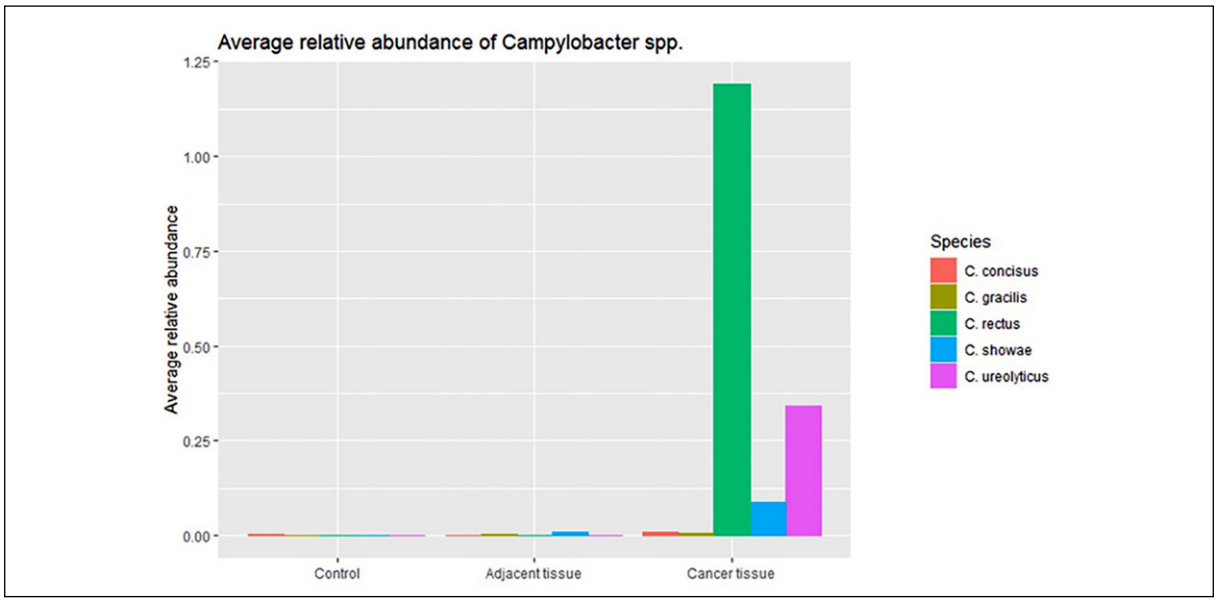

Figure 3. Showing the average relative abundance of *Campylobacter* spp.

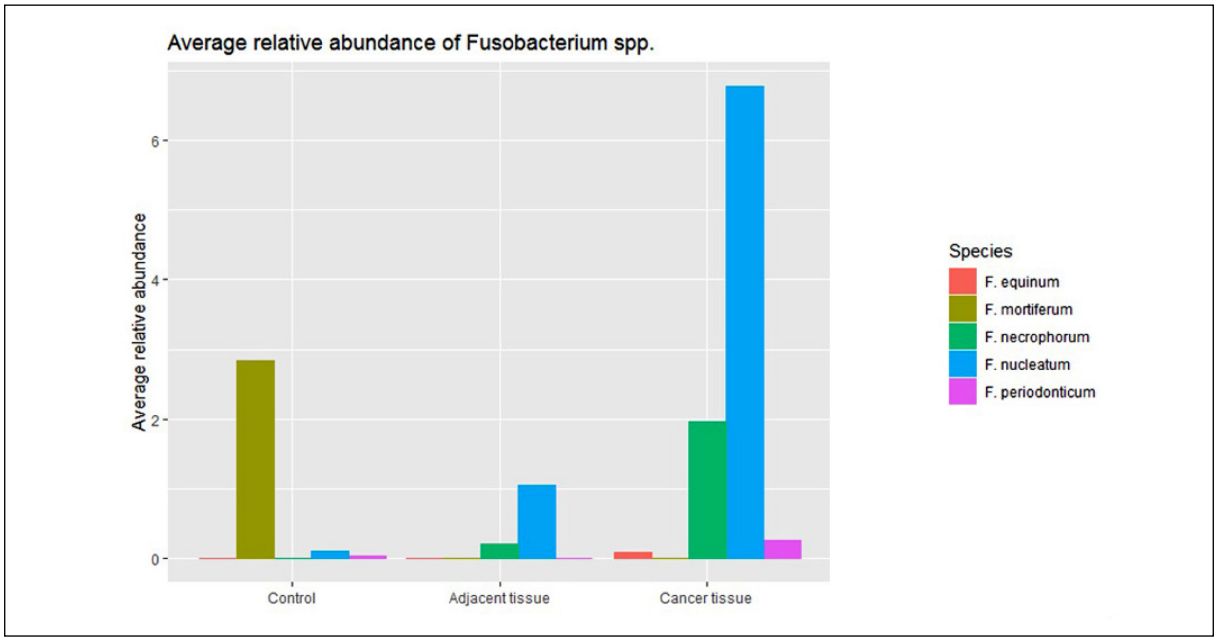

Figure 4. The relative abundance of *Fusobacterium* spp. from the biopsies in cancer and control patients. Each column represents the *Fusobacterium* spp. found in the collected group of biopsies.

Abundance of *Fusobacterium* spp

The microbiome analysis of the *Fusobacterium* spp. demonstrates the abundance of *Fusobacterium* spp. found in each group of biopsies. The bar diagram illustrates the significant differences in species found in the control group versus the cancer patients respectively, adjacent tissue and cancer tissue. The control group displays an increased abundance of *F. mortiferum* and low levels of *F. nucleatum*. Compared to the cancer tissue biopsies, which demonstrated greater abundance of *F. nucleatum* and *F. necrophorium.*

Abundance of *Helicobacter* spp.

The microbiome analysis of the *Helicobacter spp*. found one out of sixty samples with *helicobacters.* The biopsy is from cancer tissue and found *H. pullorum* and *H. pylori* as illustrated in Figure 5.

18s rDNA Procaryote Microbiome

In contrast to what Hansen et al [53] found in gastric biopsies, no difference in the procaryote microbiome was found between CRC patients and patients without cancer.

DISCUSSION

Fusobacterium spp.

Fusobacterium spp. were found in each group of biopsies, from the control patients to the cancer patients. The results presented in Figure 2. display the significant difference of *Fusobacterium* spp. found in the various groups: cancer tissue, adjacent tissue, and control tissue. We identified the highest abundance of *F. mortiferum* in the control patients, which was higher than in the adjacent tissue or the cancer tissue. These findings

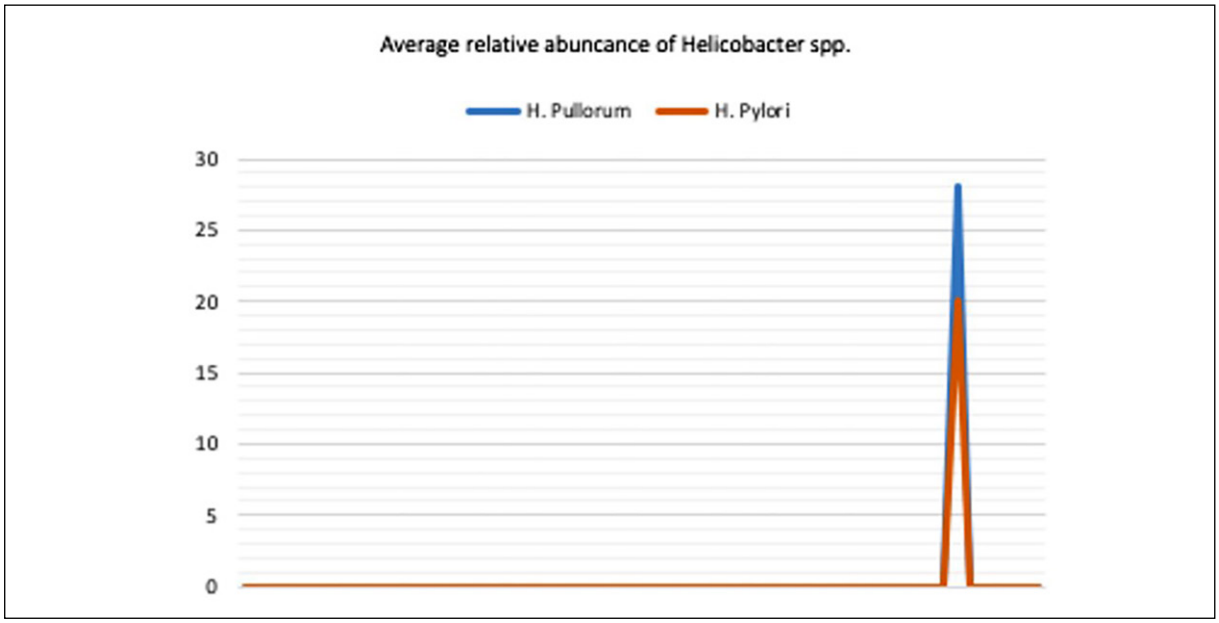

Figure 5. The relative abundance of *Helicobacter* spp. from the biopsies in cancer and control patients. The diagram illustrates one sample from cancer tissue that found *Helicobacter* spp.

differ from the genomic analysis by Kostic et al[54] who found significant phylotypes similar to *F. mortiferum* as well as *F. nucleatum* and *F. necrophorum* in colorectal tissue. Furthermore, Liang et al[55] identified a significant correlation between *F. mortiferum* and different metabolites, and highlights 2-Deoxy-D-Ribose, which may induce tumor angiogenesis. These studies suggest an association between *F. mortiferum* and tumorigenesis. However, further details and studies are necessary to quantify the relationship as current literature consists of a small quantity of studies regarding *F. mortiferum* with inconsistent analysis.

In the adjacent tissues we identified a modest amount of *F. nucleatum* and a small portion of *F. necrophorum*, both of which displayed low levels compared to the cancer tissue biopsies, which again demonstrated greater abundance of *F. nucleatum* and *F. necrophorum*. However, it portrays interest as it illustrates the same bacterial representation identified in the cancer biopsies. The high abundance of *F. nucleatum* found in the cancer tissue is unexpected as it is regarded as an oral pathogen[56] and an atypical constituent of the gut microbiota[57]. However, there is increasing evidence suggesting the involvement of *Fusobacterium* spp. and especially *F. nucleatum* in CRC and tumorigenesis. Kostic et al[54] found an increased amount of *Fusobacterium* spp. in CRC patients and suggests how *Fusobacterium* spp. might elicit a proinflammatory response and contribute to tumorigenesis. Kostic et al[58] provided supportive findings by introducing *F. nucleatum* into mice with mutated tumor suppressor gene Apc, resulting in a significant quantity of developed colon tumors compared to other mice. Flanagan et al[29] further highlights an overrepresentation of *F. nucleatum* in CRC tissue compared to normal tissue. These studies support our findings that *Fusobacterium* and *F. nucleatum* is, to a greater extent, present in CRC compared to normal tissue. Nevertheless, further studies are needed to establish *F. nucleatums* relationship in tumorigenesis.

In this study we found greater amounts of *F. necrophorum* as well as traces of *F. eqinum* and *F. Periodonticus*; also known to be oral pathogens. It raises the question whether these oral pathogens may also be found in the intestinal tract, and whether they are related to tumorigenesis, as they were solely found in the cancer tissue and adjacent tissue. Strauss et al[59] also found *F. periodonticum* in intestinal biopsies and also question whether these *Fusobacterium* spp. are found elsewhere. Our results indicate a connection between these oral pathogens in intestinal flora and the presence of cancer. This is in accordance with the experience

that when *F. necrophorum* is found in blood cultures, CRC should be suspected. However, with the small amounts found in our biopsies, additional studies are needed to confirm these findings as well as any relation to tumorigenesis.

Campylobacter spp.

Our data show a clear overrepresentation of *Campylobacter* spp. in the cancer biopsies compared to the adjacent and control biopsies. Figure 3 illustrates a clear composition of *C. rectus*, *C. ureolyticus*, *C. showae* and traces of *C. concisus* in the cancer biopsies. The adjacent and control biopsies show little to no trace of *Campylobacter* spp. demonstrating a distinct difference in the bacterial composition. Corresponding findings were found by Warren et al[60] who highlights overrepresentation of *Fusobacterium* spp. and *Campylobacter* spp. in tumor biopsies compared to healthy controls. Furthermore, Wu et al[61] compared fecal samples from CRC group and healthy controls and found a clear overabundance of *Fusobacterium* spp. and *Campylobacter* spp. in the CRC group. Interestingly, our findings show greater abundance of *C. rectus* in the cancer biopsies. *C. rectus* is well known in periodontal diseases[62,63] and not in gastrointestinal diseases or CRC. However, *C. rectus* harbor inflammatory properties by enhancing IL-6 and IL-8 production[64]. Ning and Lenz [46] argue that IL-8 and its receptor CXCR2 are upregulated in CRC. Thus, it can be argued that *C. rectus* possesses the necessary properties to stimulate chemokine production and thereby promote tumor progression in CRC. Nonetheless, these are new findings, and further studies are needed to establish the precise connection. Furthermore, the relative high abundance of *C. ureolyticus* found in the cancer biopsies show similar properties as *C. rectus*. *C. ureolyticus* is commonly found in periodontal disease[65] but is also found in gastrointestinal diseases such as IBD and colitis[66], which differ from *C. rectus*. Moreover, Burgos-Portugal et al[67] found that *C. ureolyticus* produces significantly high levels of IL-8 and generate an inflammatory response, compared to healthy controls, which is identical to *C. recuts*. However, they found pro inflammatory response in the epithelial cells of the gastrointestinal tract, which is not known for *C. rectus*. Also, *C. ureolyticus* possesses the ability to translocate through the intestinal epithelial cells with and without pre-existing inflammation. It is evident that *C. ureolyticus* elicit pro carcinogenic properties which supports the possibility of *C. ureolyticus* involvement in CRC development.

Finally, we found low abundance of *C. showae* in the cancer biopsies. *C. showae* is known to be found in IBD, Chrons disease, intra orbital abscess and blood[40,43,62], and it is therefore interesting to find *C. showae* in CRC. Consistent with this, Warren et al[60] found *Campylobacter* species, predominantly *C. showae* in co-aggregation with *Fusobacterium* spp. in CRC tissues. Furthermore, Wang et al[68] found *Fusobacterium* and *Campylobacter* species as well as *streptococcus* in the tumor biopsies compared to off tumor site. Interestingly, these findings correspond to our results with the co-occurrence of *Fusobacterium* spp. and *Campylobacter* spp. in the cancer biopsies. This presents the possibility that *Campylobacter* spp. and *Fusobacterium* spp. may either grow together or work in co-aggregation to initiate or development CRC.

Helicobacter spp.

Our analysis of the *Helicobacter* spp. found one cancer tissue biopsy with the presence of *H. pullorum* and *H. pylori,* as illustrated in Figure 5. We expected to find a higher prevalence of *Helicobacter* spp. in the biopsies. We hypothesized that the carcinogenic genes found in *H. pylori* also could be present in other intestinal *Helicobacter* spp. and therefore show a greater abundance in our cancer biopsies. The genus *Helicobacter* has a variety of mechanisms, and thus might possess the components for carcinogenesis. *H. pylori* has a well-known carcinogenic profile, and an increasing number of studies conclude that NHPH could influence intestinal cancer development[69]. Nevertheless, our data were unable to demonstrate sufficient evidence of such association. Further studies are needed to understand NHPH involvement in intestinal cancers and carcinogenesis.

CONCLUSIONS

This study illustrates a significant difference in the bacterial composition in cancer tissue compared to control tissue. It is becoming increasingly clear that there is a connection between the microbiota and colorectal carcinogenesis. Our data showed that there is statistical significance between the bacterial microbiota found in cancer tissue, adjacent tissue, and control tissue p=.001. The cancer tissue showed great abundance of *C. rectus*, *C. showae*, *C. ureolyticus* as well as *F. nucleatum* and *F. necrophorum*. This composition was solely found in the cancer tissue and to some extent in the adjacent tissue. Therefore, it can be argued that these *Campylobacter* spp. and *Fusobacterium* spp. either solely or in co-aggregation may cause or promote CRC. However, the potential pathogenic interaction between these species is not currently understood and we are therefore unable to conclude whether these species acted solely or simultaneously. Nevertheless, this study has presented evidence of a distinct microbiome in cancer tissue, which advocates for further research to characterize the composition of this microbiota and its role in carcinogenesis.

Conflict of Interest

The authors declared no conflict of interest.

Availability of Data and Materials

The datasets used during the current study are available from the corresponding author on reasonable request.

Author Contributions

LPA, KF, SS, KK, JK, LK – concept development, critical review of the manuscript, LJ, MU, JS – collection of biological samples and clinical data. KF, MS – are doing the microbiome analysis. AMO – Preparing the manuscript and doing the Epsilonproteobacter and Helicobacter PCR.

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Statement of Ethics

All patients participating in the study have signed an informed consent form.

REFERENCES

1. Arnold M, Sierra MS, Laversanne M, Soerjomataram I, Jemal A, Bray F. Global patterns and trends in colorectal cancer incidence and mortality. Gut 2017; 66: 683-691.
2. Chu KM. Epidemiology and Risk Factors of Colorectal Cancer. In S. L. Gearhart N. Ahuja (Eds.), Early Diagnosis and Treatment of Cancer Series: Colorectal Cancer 2011; 1-11.
3. Niksic M, Forbes LJL. Awareness of Colorectal Cancer: Recognition of Symptoms and Risk Factors by Socio-demographic Characteristics. In L. Olsson (Ed.), Timely Diagnosis of Colorectal Cancer 2018; 1-20.
4. Houghton JM. Introduction. In J. Houghton (Ed.), Helicobacter Species: Methods and Protocols 2012; 1-5.
5. Car LT, Papachristou N, Urch C, Majeed A, El–Khatib M, Aylin P, Atun R, Car J, Vincent C. Preventing delayed diagnosis of cancer: Clinicians' views on main problems and solutions. J Glob Health 2016; 6: 020901.

6. Jass JR. Colorectal polyposes: From phenotype to diagnosis. Pathology - Research and Practice. Pathol Res Pract 2008; 204: 431-447.
7. Dionigi G, Annoni M, Rovera F, Boni L, Villa F, Castano P, Bianchi V, Dionigi R. Primary colorectal lymphomas: Review of the literature. Surg Oncol 2007; 16: 169-171.
8. Leslie A, Carey FA, Pratt NR, Steele RJ. The colorectal adenoma–carcinoma sequence. Br J Surg 2002; 89: 845-860.
9. Ponz de Leon M. The Causes of Colorectal Cancer. In M. Ponz de Leon (Ed.), Colorectal Cancer 2002; 1-22.
10. Sun T, Liu S, Zhou Y, Yao Z, Zhang D, Cao S, Wei Z, Tan B, Li Y, Lian Z, Wang S. Evolutionary biologic changes of gut microbiota in an 'adenoma-carcinoma sequence' mouse colorectal cancer model induced by 1, 2-Dimethylhydrazine. Oncotarget 2017; 8: 444-457.
11. Gagnière J, Raisch J, Veziant J, Barnich N, Bonnet R, Buc E, Bringer MA, Pezet D, Bonnet M. Gut microbiota imbalance and colorectal cancer. World J Gastroenterol 2016; 22: 501-518.
12. Bullman S, Pedamallu CS, Sicinska E, Clancy TE, Zhang X, Cai D, Neuberg D, Huang K, Guevara F, Nelson T, Chipashvili O, Hagan T, Walker M, Ramachandran A, Diosdado B, Serna G, Mulet N, Landolfi S, Cajal S, Fasani R, Aguirre AJ, Ng K, Élez E, Ogino S, Tabernero J, Fuchs CS, Hahn WC, Nuciforo P, Meyerson M. Analysis of Fusobacterium persistence and antibiotic response in colorectal cancer. Science 2017; 358: 1443-1448.
13. Mizutani S, Yamada T, Yachida S. Significance of the gut microbiome in multistep colorectal carcinogenesis. Cancer Sci 2020; 111: 766-773.
14. Saus E, Iraola-Guzmán S, Willis JR, Brunet-Vega A, Gabaldón T. Microbiome and colorectal cancer: Roles in carcinogenesis and clinical potential. Mol Aspects Med 2019; 69: 93-106.
15. Murphy N, Moreno V, Hughes DJ, Vodicka L, Vodicka P, Aglago EK, Gunter MJ, Jenab M. Lifestyle and dietary environmental factors in colorectal cancer susceptibility. Mol Aspects Med 2019; 69: -9.
16. Mehta RS, Nishihara R, Cao Y, Song M, Mima K, Qian ZR, Nowak JA, Kosumi K, Hamada T, Masugi Y, Bullman S, Drew DA, Kostic AD, Fung TT, Garrett WS, Huttenhower C, Wu K, Meyerhardt JA, Zhang X, Willett WC, Giovannucci EL, Fuchs CS, Chan AT, Ogino S. Association of Dietary Patterns with Risk of Colorectal Cancer Subtypes Classified by Fusobacterium nucleatum in Tumor Tissue. JAMA Oncol 2017; 3: 921-927.
17. Chen GY. The Role of the Gut Microbiome in Colorectal Cancer. Clin Colon Rectal Surg 2018; 31: 192-198.
18. Gao Z, Guo B, Gao R, Zhu Q, Qin H. Microbiota disbiosis is associated with colorectal cancer. Front Microbiol 2015; 6: 20.
19. Thursby E, Juge N. Introduction to the human gut microbiota. Biochem J 2017; 474: 1823-1836.
20. Lam SY, Yu J, Wong SH, Peppelenbosch MP, Fuhler GM. The gastrointestinal microbiota and its role in oncogenesis. Best Pract Res Clin Gastroenterol 2017; 31: 607-618.
21. David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, Ling AV, Devlin AS, Varma Y, Fischbach MA, Biddinger SB, Dutton RJ, Turnbaugh PJ. Diet rapidly and reproducibly alters the human gut microbiome. Nature 2014; 505: 559-563.
22. Filippo CD, Cavalieri D, Paola MD, Ramazzotti M, Poullet JB, Massart S, Collini S, Pieraccini G, Lionetti P. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proc Natl Acad Sci U S A 2010; 107: 14691-14696.
23. Song M, Chan AT. Environmental Factors, Gut Microbiota, and Colorectal Cancer Prevention. Clin Gastroenterol Hepatol 2019; 17: 275-289.
24. Marchesi JR, Dutilh BE, Hall N, Peters WH, Roelofs R, Boleij A, Tjalsma H. Towards the Human Colorectal Cancer Microbiome. PLoS One 2011; 6: e20447.
25. Pasquereau-Kotula E, Martins M, Aymeric L, Dramsi S. Significance of Streptococcus gallolyticus subsp. gallolyticus Association with Colorectal Cancer. Front Microbiol 2018; 9: 614.
26. Rubinstein MR, Wang X, Liu W, Hao Y, Cai G, Han YW. Fusobacterium nucleatum Promotes Colorectal Carcinogenesis by Modulating E-Cadherin/β-Catenin Signaling via its FadA Adhesin. Cell Host Microbe 2013; 14: 195-206.
27. Yang Y, Weng W, Peng J, Hong L, Yang L, Toiyama Y, Gao R, Liu M, Yin M, Pan C, Li H, Guo B, Zhu Q, Wei Q, Moyer MP, Wang P, Cai S, Goel A, Qin H, Ma Y. Fusobacterium nucleatum Increases Proliferation of Colorectal Cancer Cells and Tumor Development in Mice by Activating Toll-Like Receptor 4 Signaling to Nuclear Factor–κB, and Up-regulating Expression of MicroRNA-21. Gastroenterology 2017; 152: 851-866.
28. Winter SE, Lopez CA, Bäumler AJ. The dynamics of gut-associated microbial communities during inflammation. EMBO Rep 2013; 14: 319-327.
29. Flanagan L, Schmid J, Ebert M, Soucek P, Kunicka T, Liska V, Bruha J, Neary P, Dezeeuw N, Tommasino M, Jenab M, Prehn JHM, Hughes DJ. Fusobacterium nucleatum associates with stages of colorectal neoplasia development, colorectal cancer and disease outcome. Eur J Clin Microbiol Infect Dis 2014; 33: 1381-1390.
30. Mima K, Nishihara R, Qian ZR, Cao Y, Sukawa Y, Nowak JA, Yang J, Dou R, Masugi Y, Song M, Kostic AD, Giannakis M, Bullman S, Milner DA, Baba H, Giovannucci EL, Garraway LA, Freeman GJ, Dranoff G, Garrett WS, Huttenhower C, Meyerson M, Meyerhardt JA, Chan AT, Fuchs CS, Ogino S. Fusobacterium nucleatum in colorectal carcinoma tissue and patient prognosis. Gut 2016; 65: 1973-1980.
31. Deng Q, Wang C, Yu K, Wang Y, Yang Q, Zhang J, Xu X. Streptococcus bovis Contributes to the Development of Colorectal Cancer via Recruiting CD11b+TLR-4+ Cells. Med Sci Monit 2020; 26: e921886.
32. Chew SS, Lubowski DZ. Clostridium septicum and malignancy. ANZ J Surg 2001; 71: 647-649.
33. Mirza NN, McCloud JM, Cheetham MJ. Clostridium septicum sepsis and colorectal cancer—A reminder. World J Surg Oncol 2009; 7: 73.
34. Høiby N, Skinhøj P. Compendium in microbiology and infectious medicine (1st ed.). FADL´s publisher 2015; 84.

35. Könönen E, Conrads G, Nagy E. Bacteroides, Porphyromonas, Prevotella, Fusobacterium, and Other Anaerobic Gram-Negative Rods. Manual of Clinical Microbiology 2015; 967-993.
36. Tahara T, Yamamoto E, Suzuki H, Maruyama R, Chung W, Garriga J, Jelinek J, Yamano HO, Sugai T, An B, Shureiqi I, Toyota M, Kondo Y, Estécio MR, Issa JP. Fusobacterium in Colonic Flora and Molecular Features of Colorectal Carcinoma. Cancer Res 2014; 74: 1311-1318.
37. Kumar V, Abbas AK, Aster JC. Robbins Basic Pathology. Elsevier 2017; 18: 710-712.
38. Peng X, Zhou L, Gong Y, Song Z, He L, Lin S, Zhang J. Non-pylori Helicobacters (NHPHs) Induce Shifts in Gastric Microbiota in Helicobacter pylori-Infected Patients. Front Microbiol 2017; 8: 1038.
39. Shang FM, Liu HL. Fusobacterium nucleatum and colorectal cancer: A review. World J Gastrointest Oncol 2018; 10: 71-81.
40. Kaakoush NO, Castaño-Rodríguez N, Mitchell HM, Man SM. Global Epidemiology of Campylobacter Infection. Clin Microbiol Rev 2015; 28: 687-720.
41. Man SM. The clinical importance of emerging Campylobacter species. Nat Rev Gastroenterol Hepatol 2011; 8: 669-685.
42. Costa D, Iraola G. Pathogenomics of Emerging Campylobacter Species. Clin Microbiol Rev 2019; 32: e00072-18.
43. Man SM, Kaakoush NO, Leach ST, Nahidi L, Lu HK, Norman J, Day AS, Zhang L, Mitchell HM. Host Attachment, Invasion, and Stimulation of Proinflammatory Cytokines by Campylobacter concisus and Other Non-Campylobacter jejuni Campylobacter Species. J Infect Dis 2010; 202: 1855-1865.
44. Tjalsma H, Boleij A, Marchesi JR, Dutilh BE. A bacterial driver–passenger model for colorectal cancer: Beyond the usual suspects. Nat Rev Microbiol 2012; 10: 575-582.
45. Singh S, Sadanandam A, Singh RK. Chemokines in tumor angiogenesis and metastasis. Cancer Metastasis Rev 2007; 26: 453-467.
46. Ning Y, Lenz HJ. Targeting IL-8 in colorectal cancer. Expert Opin Ther Targets 2012; 16: 491-497.
47. Kupcinskas J, Hold GL. Other Helicobacters and the gastric microbiome. Helicobacter 2018; 23: e12521.
48. Amieva M, Peek RM Jr. Pathobiology of Helicobacter pylori–Induced Gastric Cancer. Gastroenterology 2016; 150: 64-78.
49. Fox JG. The non-H pylori helicobacters: Their expanding role in gastrointestinal and systemic diseases. Gut 2002; 50: 273-283.
50. Belzer C, Stoof J, Beckwith CS, Kuipers EJ, Kusters JG, van Vliet AHM. Differential regulation of urease activity in Helicobacter hepaticus and Helicobacter pylori. Microbiology 2005; 151: 3989-3995.
51. Monographs available – IARC Monographs on the Identification of Carcinogenic Hazards to Humans. (n.d.). Retrieved March 18, 2022, from https://monographs.iarc.who.int/monographs-available/
52. Spiegelhauer MR, Kupcinskas J, Johannesen TB, Urba M, Skieceviciene J, Jonaitis L, Frandsen TH, Kupcinskas L, Fuursted K, Andersen LP. Transient and Persistent Gastric Microbiome: Adherence of Bacteria in Gastric Cancer and Dyspeptic Patient Biopsies after Washing. J Clin Med 2020; 9: 1882.
53. Hansen ABR, Johannesen TB, Spiegelhauer MR, Kupcinskas J, Urba M, Skieceviciene J, Jonaitis L, Frandsen TH, Kupcinskas L, Fuursted K, Andersen LP. Distinct composition and distribution of the gastric mycobiota observed between dyspeptic and gastric cancer patients evaluated from gastric biopsies. Microb Health Dis 2020; 2: e340.
54. Kostic AD, Gevers D, Pedamallu CS, Michaud M, Duke F, Earl AM, Ojesina AI, Jung J, Bass AJ, Tabernero J, Baselga J, Liu C, Shivdasani RA, Ogino S, Birren BW, Huttenhower C, Garrett WS, Meyerson M. Genomic analysis identifies association of Fusobacterium with colorectal carcinoma. Genome Res 2012; 22: 292-298.
55. Liang S, Mao Y, Liao M, Xu Y, Chen Y, Huang X, Wei C, Wu C, Wang Q, Pan X, Tang W. Gut microbiome associated with APC gene mutation in patients with intestinal adenomatous polyps. Int J Biol Sci 2020; 16: 135-146.
56. Castellarin M, Warren RL, Freeman JD, Dreolini L, Krzywinski M, Strauss J, Barnes R, Watson P, Allen-Vercoe E, Moore RA, Holt RA. Fusobacterium nucleatum infection is prevalent in human colorectal carcinoma. Genome Res 2012; 22: 299-306.
57. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, Nielsen T, Pons N, Levenez F, Yamada T, Mende DR, Li J, Xu J, Li S, Li D, Cao J, Wang B, Liang H, Zheng H, Xie Y, Tap J, Lepage P, Bertalan M, Batto JM, Hansen T, Le Paslier D, Linneberg A, Nielsen HB, Pelletier E, Renault P, Sicheritz-Ponten T, Turner K, Zhu H, Yu C, Li S, Jian M, Zhou Y, Li Y, Zhang X, Li S, Qin N, Yang H, Wang J, Brunak S, Doré J, Guarner F, Kristiansen K, Pedersen O, Parkhill J, Weissenbach J, Bork P, Ehrlich SD, Wang J. A human gut microbial gene catalogue established by metagenomic sequencing. Nature 2010; 464: 59-65.
58. Kostic AD, Chun E, Robertson L, Glickman JN, Gallini CA, Michaud M, Clancy TE, Chung DC, Lochhead P, Hold GL, El-Omar EM, Brenner D, Fuchs CS, Meyerson M, Garrett WS. Fusobacterium nucleatum Potentiates Intestinal Tumorigenesis and Modulates the Tumor-Immune Microenvironment. Cell Host Microbe 2013; 14: 207-215.
59. Strauss J, White A, Ambrose C, McDonald J, Allen-Vercoe E. Phenotypic and genotypic analyses of clinical Fusobacterium nucleatum and Fusobacterium periodonticum isolates from the human gut. Anaerobe 2008; 14: 301-309.
60. Warren RL, Freeman DJ, Pleasance S, Watson P, Moore RA, Cochrane K, Allen-Vercoe E, Holt RA. Co-occurrence of anaerobic bacteria in colorectal carcinomas. Microbiome 2013; 1: 16.
61. Wu N, Yang X, Zhang R, Li J, Xiao X, Hu Y, Chen Y, Yang F, Lu N, Wang Z, Luan C, Liu Y, Wang B, Xiang C, Wang Y, Zhao F, Gao GF, Wang S, Li L, Zhang H, Zhu B. Dysbiosis Signature of Fecal Microbiota in Colorectal Cancer Patients. Microb Ecol 2013; 66: 462-470.
62. Liu F, Ma R, Wang Y, Zhang L. The Clinical Importance of Campylobacter concisus and Other Human Hosted Campylobacter Species. Front Cell Infect Microbiol 2018; 8: 243.

63. Könönen E, Paju S, Pussinen PJ, Hyvönen M, Tella PD, Suominen-Taipale L, Knuuttila M. Population-Based Study of Salivary Carriage of Periodontal Pathogens in Adults. J Clin Microbiol 2007; 45, 2446-2451.
64. Dongari-Bagtzoglou AI, Ebersole JL. Production of inflammatory mediators and cytokines by human gingival fibroblasts following bacterial challenge. J Periodontal Res 1996; 31: 90-98.
65. Duerden BI, Goodwin L, O'Neil TC. Identification of Bacteroides species from adult periodontal disease. J Med Microbiol 1987; 24: 133-137.
66. Bullman S, Lucid A, Corcoran D, Sleator RD, Lucey B. Genomic Investigation into Strain Heterogeneity and Pathogenic Potential of the Emerging Gastrointestinal Pathogen Campylobacter ureolyticus. PLoS One 2013; 8: e71515.
67. Burgos-Portugal JA, Kaakoush NO, Raftery MJ, Mitchell HM. Pathogenic Potential of Campylobacter ureolyticus. Infect and Immun 2011; 80: 883-890.
68. Wang Z, Ren R, Yang Y. Mucosa microbiome of gastric lesions: Fungi and bacteria interactions. Prog Mol Biol Transl Sci 2020; 171: 195-213.
69. Kupcinskas J, Hold GL. Other Helicobacters and the gastric microbiome. Helicobacter 2018; 23: e12521.