Tesis Doctoral

ROLE OF INTESTINAL MICROBIOTA COMPOSITION IN COLORECTAL CANCER AND IN THE RESPONSE TO NEOADJUVANT RADIOCHEMOTHERAPY PREVIOUS TO SURGERY

Lidia Sánchez Alcoholado

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Facultad de Medicina

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Directores:
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DECLARACIÓN DE AUTORÍA Y ORIGINALIDAD DE LA TESIS PRESENTADA PARA OBTENER EL TÍTULO DE DOCTOR

D./Dña LIDIA SÁNCHEZ ALCOHOLADO

Estudiante del programa de doctorado BIOMEDICINA, INVESTIGACIÓN TRASLACIONAL Y NUEVAS TECNOLOGÍAS EN SALUD de la Universidad de Málaga, autor/a de la tesis, presentada para la obtención del título de doctor por la Universidad de Málaga, titulada: ROLE OF INTESTINAL MICROBIOTA COMPOSITION IN COLORECTAL CANCER AND IN THE RESPONSE TO NEOADJUVANT RADIOCHEMOTHERAPY PREVIOUS TO SURGERY

Realizada bajo la tutorización de FERNANDO CARDONA DÍAZ y dirección de MARÍA ISABEL QUEIPO ORTUÑO Y FERNANDO CARDONA DÍAZ (si tuviera varios directores deberá hacer constar el nombre de todos)

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Por la presente los doctores, **Dª. María Isabel Queipo Ortuño**, Investigadora Miguel Servet, en el Instituto de Investigación Biomédica de Málaga (IBIMA), y **D. Fernando Cardona Díaz**, Profesor Titular, adscrito al Área de Bioquímica y Biología Molecular del departamento de Especialidades Quirúrgicas, Bioquímica e Inmunología de la Facultad de Medicina de la Universidad de Málaga.

CERTIFICAN que Dª. Lidia Sánchez Alcoholado ha obtenido y estudiado personalmente bajo nuestra dirección y supervisión los datos necesarios para la realización de su Tesis Doctoral, titulada: *"Role of intestinal microbiota composition in colorectal cancer and in the response to neoadjuvant radiochemotherapy previous to surgery"*, que consideramos tiene el contenido y rigor científico necesario para ser sometido al superior juicio de la Comisión que nombre la Universidad de Málaga para optar a grado de Doctor.

Y para que conste, en cumplimiento de las disposiciones vigentes, se expide el presente certificado en:

Málaga 26 de febrero de 2021

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INTRODUCTION

1. COLORECTAL CANCER

1.1. EPIDEMIOLOGY

We live in an era in which access to health care has increased and the diagnosis and treatment of disease has improved, which has had an impact on average life expectancy in most regions of the world. As a result of these medical improvements, disease-associated mortality has generally decreased, however, cancer-associated mortality has increased in recent decades. According to the latest data from the World Health Organization (WHO), cancer is the second leading cause of death in the world, it is estimated that in 2018, cancer caused 9.6 million deaths, being responsible for one of every six deaths in the world.

Every year more than a quarter of a million people around the world are affected by colorectal cancers (CRC). In industrialized nations the risk of developing CRC is approximately 5%, this probability increases up to 20% in the case of developing an adenoma or a non-cancerous colon tumor [1].

When the disease is localized, effective treatment success rates range from 70–90%; however, advanced CRC has a high mortality rate, consistently ranking in the top three causes of cancer-related death worldwide.

The geographical location is an important factor to take into account in the study of the appearance of new cases of cancer, since it determines different lifestyles and cultures, as well as differences for some types of cancers, between men and women [2] or between different ethnic and racial groups. Areas of low incidence have been seen in the study of CRC, such as Africa and parts of Asia, where a risk range of approximately 5 per 100,000 is estimated, while areas of high incidence such as the United States, Western Europe,

Australia and New Zealand can reach a range of 40 per 100,000 [3], being higher in Japan [4]. However, the increased risk of CRC in some countries does not necessarily correspond to a higher mortality rate. The United States has a high incidence rate of CRC, while it has a low mortality. In Brazil, India, China and some African countries the opposite occurs, the incidence of suffering from the disease is low while the mortality rate remains high [5]. Access to treatment is very important in reducing the mortality rate, however the implementation of screening programs has been a determining factor for early detection, as early as 1992 the first screening programs were established in Italy and Israel [6]. The Spanish CRC incidence rate exceeds the European average, being 30.4 per 100,000, with the mortality rate remaining at the average, 13.3 per 100,000 [7, 8]. In Spain, CRC is the first most common type of cancer and the second in mortality, if men and women are studied together [9].

In the difference between males and females, the CRC seems to have a higher incidence in males [10] especially at younger ages even having lifestyles similar to those of females. There are studies that affirm that the risk of developing this type of cancer in males and females is equal in later ages [2]. However, other studies have estimated a small statistical difference in the possibility of developing colorectal adenomas for males with respect to females, being the mean age of the groups 65 years [11]. And according to the 2018 data from GLOBOCAN, both the incidence and mortality rates due to CRC worldwide are higher for males than for females, being more evident above 50 years [10].

The development of cancer in general depends on a wide range of factors. The human large intestine is a very common place for adenocarcinomas development, but it is also the place where one of the most densely populated microbial ecosystems exists on the planet. Although the appearance of CRC is highly heterogeneous from the genetic and

epigenetic point of view, the possible role of intestinal microorganisms in colorectal carcinogenesis should not be ignored [12].

1.2. RISK FACTORS

The etiology of CRC is very heterogeneous, age is considered to be a highly influential factor in the onset of the disease [9], but the combination of genetic alterations and environmental factors is which confers it variability. Most CRC cases are sporadic in patients with no family history of the disease, occurring in three-quarters of cases [13].

The modifiable causes that influence the appearance of CRC are identifiable and can be prevented, since these causes are associated with cultural and social factors [14, 15], so that a healthy lifestyle that avoids those known factors would decrease the risk of the appearance of this disease (**Figure 1**).
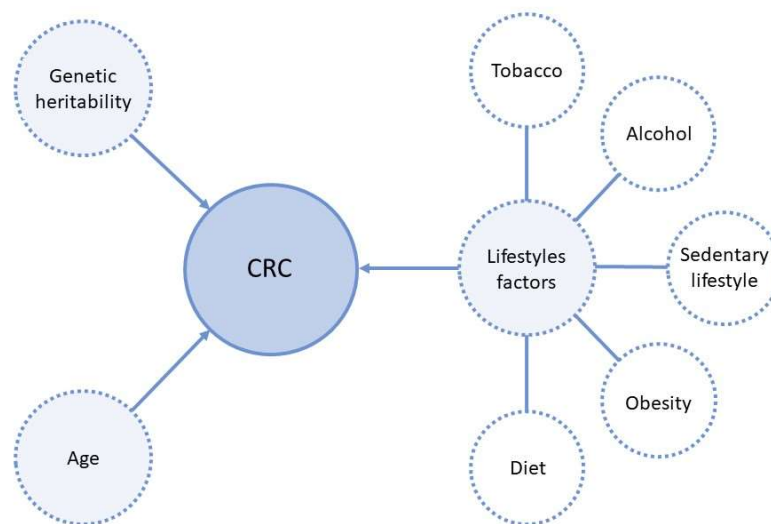
Figure 1. Scheme of the main risk factors that affect the development of colorectal cancer (CRC). It includes the lifestyle habits that most affect CRC.

1.2.1. AGE

The older the age, the greater the probability of developing CRC, greatly increasing this probability after 50 years of age. However, in recent times, studies have been carried out in different regions and it has been seen that patients under 50 years of age have shown a significantly higher probability [16, 17]. Patients under 50 years of age are less likely to have CRC, however, there are studies that show an increase in cases diagnosed at those ages, which tend to be in advanced stages. The survival prognosis is significantly worse when the diagnosis of the disease occurs at an advanced stage regardless of age [18].

The fact that advanced cases of CRC are more commonly diagnosed in patients under 50 can be attributed to genetic predisposition, previous misdiagnoses, or overlook of the symptoms of the disease [10,19]. However, the increase in diagnosed cases of CRC in those under 50 years of age, regardless of stage, usually associated with risk factors related to lifestyle, such as the dietary factors [20].

1.2.2. GENETIC FACTORS

The majority of CRC cases are sporadic and only a small proportion of them occur as a consequence of genetic inheritance, as a result of familial adenomatous polyposis (<1%), MYH gene associated polyposis (< 1%), or hereditary CRC without polyposis, also known as Lynch syndrome (2-5%) [21]. The risk of developing this disease is higher if there is a family history. If the number of family members with a positive diagnosis for the disease increases, the risk of developing CRC also increases [13]. Regardless of age, people with inherited cancer syndromes have a higher risk of CRC than the rest of the population because they have germline mutations for high penetrance genes that are usually autosomal dominant, thus that people with no family history could develop hereditary cancer syndrome [10].

A definite characteristic of CRC is genomic instability [22] that includes genetic or genomic changes that can be a point mutation or chromosomal rearrangement [23].

The most frequent acquired genetic alterations that appear in CRC are due to chromosomal instability, which includes numeric or subchronic chromosomal aberrations, loss of heterozygosity, and amplifications [24]. The loss of tumor suppressor genes is the main negative effect that will lead to carcinogenesis. Various genes such as Adenomatous polyposis coli gene (APC), Kristen rat sarcoma virus gen (KRAS), Tumor protein 53 gene (TP53), Phosphatidylinositol-4,5-Biphosphate 3- Kinase catalytic subunit alpha gene (PI3KCA) and SMAD family member 4 gene (SMAD4) or Deleted in pancreatic cancer 4 gene (DPC4) play an important role in the development of CRC [24]. Poor chromosomal segregation can lead to mutations in the APC gene that activate the Wnt signaling pathway, a key step in the initiation of 80% of CRCs [22]. When the TP53 gene is mutated, a cell cycle checkpoint is lost and uncontrolled cell proliferation occurs and drives the progression of carcinogenesis [25, 26].

Microsatellite instabilities are somatic alterations in specific regions of DNA called microsatellites which are short sequences of nucleotide bases, repeated multiple times [24]. The repair of mismatches during replication results in the accumulation of DNA mutations mainly in the DNA fragments of microsatellites with repetitive nucleotide sequence. This microsatellite instability can be identified using the polymerase chain reaction (PCR) test, comparing normal and tumor DNA from the same patient [13]. Lynch syndrome, the most common inherited CRC syndrome, is characterized by a mutation in one of the genes responsible for repairing the DNA mismatch (MutL Homolog 1 (MLH1), Epithelial cellular adhesion molecule (EPCAM), Postmeiotic Segregation Increased 2 (PMS2), MutS Homolog 2 (MSH2), or MutS Homolog 6 (MSH6)) [13]. The

accumulation of mating alterations occurs mainly in the microsatellite regions of DNA with repeating nucleotide sequences.

Epigenetic alterations appear commonly in polyps and CRC, probably in association with carcinogenesis promoting mutations [27, 28]. DNA methylation is a modification of DNA that has been identified as the third route of carcinogenesis [9]. The CpG (Dinucleotide Citosin-Guanine) rich regions or CpG islands are the regions of DNA that are affected by methylation. Hypermethylation can cause transcriptional silencing [29] including tumor suppressor genes [9, 24]. Hypomethylation of repetitive genetic elements is associated with the activation of oncogenes and genomic instability [30].

1.2.3. LIFESTYLE FACTORS

The development of CRC is associated, in addition to genetic predisposition, to a series of environmental factors, which are identifiable and modifiable, which would help to prevent the appearance of CRC to some extent. These known causes are widely related to cultural and social factors [14, 15] so that the prevalence of these modifiable factors that are related to lifestyle, could explain, to a large extent, the variation in the geographic and socioeconomic status of the onset of this disease [31].

Thus, genetics individually influence the appearance of CRC, while factors associated with lifestyle affect the incidence of the population suffering from the disease. This has been seen in studies with migratory populations, in which it has been observed that the incidence rate of CRC in the migrating population varied rapidly to reach the rate of the host population [1, 32, 33].

The identified factors whose modification can help prevent the appearance of CRC are: smoking, alcohol consumption, obesity, sedentary lifestyle, high consumption of red and processed meats and low fiber intake.

- **Tobacco**

Tobacco is associated with up to 12% of deaths caused by CRC [34]. The carcinogens present in tobacco are carried by smoke and can reach the colonic mucosa through the circulatory system or directly by ingesting them, where they can induce genetic and epigenetic aberrations responsible for the formation of CRC [35]. As previously mentioned, CRC is a very heterogeneous disease, both in its origin and its development, there are different subtypes which are not equally influenced by factors. In this case, the tobacco, (responsible for the increase in the rate of formation and development of adenomatous polyps that are the precursor lesion of CRC) [36, 37], in addition to presenting differences in anatomical subtypes. In a study that included patients from 10 European countries an increased risk of developing proximal colon cancer was observed [10].

Epigenetic alterations are strongly associated with smoking-induced neoplasms, which was determined in a study in which the methylation of the human genome was studied and the presence of DNA methylation patterns appeared in smokers but not in non-smokers [38]. In the same study, it was observed that the methylation patterns of ex-smokers were more similar to those of people who had never smoked, suggesting that aberrant methylation may revert to normal after smoking cessation. Remembering the existence of different subtypes of CRC, only some benefit from the short-term reversible effect of quitting smoking, reducing the risk of CRC by approximately 50% after 10 years of cessation of smoking [39]. While on the other hand, smoking also has an irreversible

effect that takes time to emerge, which is the consequence of the action of tobacco carcinogens (such as polycyclic aromatic hydrocarbons) that have an action on DNA creating adducts that damage it irreversibly although quit smoking [35].

- **Alcohol**

Currently, the consumption of alcoholic beverages is associated with an increase in the diagnosis of patients with CRC at an early age [1, 10, 40]. Some studies have shown that people with a consumption of 30 grams of alcohol per day (equivalent to 3 glasses of wine) increases the probability of the appearance of polyps in the distal colon and rectum almost twice compared to people whose consumption was less [40]. Subsequent analyzes showed that the consumption of 30 or more grams of alcohol per day carried an increased risk of developing CRC.

Although a minimum level of alcohol consumption has not been established to produce an increase in the probability of developing the disease, in a meta-analysis that included several studies with European, North American and Asian cohorts, even light consumption alcoholic beverages ($\leq$ 1 per day) was determined to produce a slightly and significantly increased risk of developing CRC compared to individuals whose alcoholic beverage consumption was occasional or not consume [41].

The risk factor for CRC associated with any type of alcoholic beverage is due to the ethanol they contain. Ethanol will give rise to metabolites, the first of which, acetaldehyde, is classified as carcinogenic to humans according to the International Agency for Research.

The ingested alcohol reaches the colonocytes through the systemic circulation, where the ethanol diffuses through the lumen of the intestine where the microbial alcohol

dehydrogenase metabolizes it, giving rise to acetaldehyde [42]. The acetaldehyde found in the lumen causes damage to the mucosa and its consequent cell proliferation to regenerate it [43]. But this molecule also enters intestinal epithelial cells where it has a toxic effect, since the low activity of acetoaldehyde dehydrogenase in the colon mucosa causes it to accumulate inside the cell [42]. The intracellular accumulation of the ethanol metabolite causes DNA damage and destroys folate, a molecule necessary for the correct synthesis and methylation of DNA, which favors colorectal carcinogenesis [43, 44].

The social relationship between alcohol consumption and smoking must also be taken into account [34]. Alcohol and tobacco when acting together increase the incidence of CRC. The presence of alcohol makes the repair of tobacco-induced DNA mutations less efficient, in addition, alcohol can act as a solvent for other carcinogenic molecules, favoring their entry into mucosal cells [45].

- **Sedentary lifestyle**

Lack of physical activity is a risk factor for CRC [46] being the third most important socio-cultural factor that affects this disease [1]. There is strong evidence that a higher level of physical activity is associated with a lower risk of various types of cancer [46-48]. And convincing evidence of the beneficial effect of physical activity on the risk of colon and breast cancer and probable evidence for other cancers [49-51]. According to the National Institutes of Health (NIH), the data that relate more physical activity with a lower risk of cancer come mainly from observational studies in which people who report their physical activity are followed for several years to later determine cancer diagnoses. Although no chance relationship is found in these types of studies, when similar results are obtained in studies with different populations a possible connection casual is evidences.

Original scientific articles and systematic reviews have demonstrated a dose–response relationship between physical activity and cancer risk [46, 49]. In the case of CRC, data from multiple epidemiological studies indicate that physical activity after a CRC diagnosis is associated with a 30% lower risk of death from CRC and a 38% lower risk of death from any cause [48]. A meta-analysis of numerous prospective studies, examining the association between physical activity of various intensity levels and the risk of developing CRC have been evaluated, showing that increased levels of physical activity considerably decrease the colon and rectal cancer risk [52]. In another meta-analysis of 126 studies, people who participated in the highest level of physical activity had a 19% lower risk of colon cancer than people with the lowest level of physical activity [53]. In a study in which individuals with different levels of activity were compared, it was observed that the appearance of adenomatous polyps was inversely and significantly associated with physical activity, producing a 16% decrease in risk in individuals with greater activity with respect to those of minor activity [40].

The frequency and intensity of physical activity are known to be inversely related to the risk of CRC, although the optimal activity pattern to reduce risk and prevent the disease is not known [54, 55]. The benefit of physical activity is associated with the accumulation of energy expenditure due to exercise, not related to diets, which allows great flexibility when choosing exercise and its intensity, which leads to a reduction in the risk of cancer of the colon the weekly hours devoted to physical activity [10]. Currently, the rhythm of life requires us to spend a large part of the time sitting, regardless of the physical activity we do, it has been estimated that for each increase of 2 hours a day that we spend sitting, the risk of CRC increases by 7% [56, 57].

The link between physical activity and reduced risk of CRC could be established through the beneficial effects of physical activity on intestinal motility, reduction of inflammation,

improvement of the immune system and the action of metabolic hormones [40, 58]. These effects could be a direct consequence of physical activity, although they are also regulated by the reduction of visceral adipose tissue [59]. The most commonly hypothesized mechanisms proposed for relationship between physical activity and reduced cancer risk are summarized in **Figure 2**.
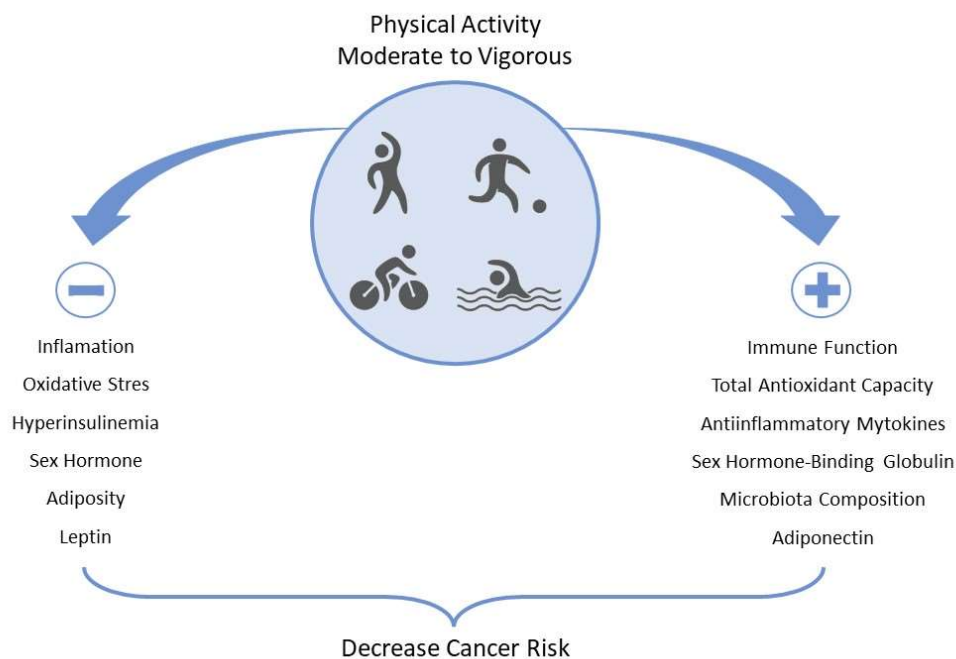
Figure 2. The effects of physical activity that advantage a decrease in the risk of cancer.

- **Obesity**

There is a positive relationship between elevated body mass index (BMI) and the development of adenomas and CRC [40]. Increasing the BMI by 5 units produces a significant 19% increase in the relative risk of developing adenomas [60], which implies a 6% increase in risk if the BMI is high. In the case of serrated polyps, when comparing individuals with low and high BMI, it was observed that the risk of polyps appearing also increased with BMI [61].

The mechanisms that control the relationship between BMI and CRC risk are associated with insulin resistance and associated hyperinsulinemia, which act on the insulin/insulin-like growth factor, inducing mitogenic and antiapoptotic signaling pathways [1, 62]. Insulin-like growth factors (IGFs) play an important role in the development and evolution of various types of cancers, not just CRC. In cancer cells, both IGF and its receptors are overexpressed in such way that the cell cycle is promoted and apoptosis is inhibited [63]. The activation of insulin receptors by insulin, initiates a sequence of consequences that trigger in the promotion of cancer. Thus, insulin stimulates its receptors and the levels of IGF-binding proteins are reduced, the levels of free IGF are increased, which activates different pathways of regulation of cell growth and proliferation such as phosphatidylinositol 3-kinase-AKT-mammalian target of rapamycin pathway and the Ras-Raf-MEK-Mitogen-Activated Protein Kinase pathway, producing a greater cellular metabolic activity that leads to higher oxidative stress and increased DNA damage [64].

Another reason that excess weight increases the risk of CRC is due to inflammation, since obesity is a low-grade chronic inflammatory state, and inflammation as such increases the probability of CRC [65].

- **Diet**

Another risk factor that increases the probability of CRC is diet. The diet includes a large amount of substances and molecules that really affect the disease, which when combined have more influence. Many studies have been done to determine the dietary patterns that could be considered healthy and unhealthy, since those considered healthy could reduce the risk of CRC by 70% [66]. A healthy pattern would include high intakes of fruits and vegetables, whole grains, nuts, legumes, fish/shellfish, and low-fat milk and dairy

products, while reducing or eliminating red and processed meats, refined grains, and products with sugars processed as desserts or sugar-sweetened beverage [67].

Comparing the dietary habits of CRC patients with healthy individuals, certain foods that can contribute to CRC have been highlighted, such as high amounts of red meat, pork, animal oils, fish, and eggs [68, 69]. Consumption of red meat, processed meat and canned foods carries an increased risk of CRC [68, 70, 71]. Therefore, moderate consumption of these foods is recommended in clinical guidelines to reduce the risk of CRC [3]. The World Cancer Research Fund (WCRF) and the American Institute for Cancer Research (AICR) conducted a meta-analysis of prospective studies showing that every 100 g/day increase in the intake of red and/or processed meat translates into in a 12% increase in the risk of CRC [72], being the effect of processed meat greater than that of red meat. Other studies showed that, when comparing individuals with high and low intakes of red/processed meat, individuals with the highest intake of red/processed meat had an increase of around 20% in the risk of adenomatous polyps [71], being similarly estimate for serrated polyps [61]. The influence of red meat and processed meat on the increased risk of CRC is estimated to be due to carcinogenic compounds derived from them, such as haem iron from red meat, exogenous N-nitroso compounds from processed meat, ionized fatty acids and secondary bile acids attributable to fat in meats, and heterocyclic amines and polycyclic aromatic hydrocarbons formed when meats are cooked at high temperatures [73-76].

On the other hand, the reduction in the risk of CRC has been associated with a high intake of fruits and vegetables, an increase in fiber and micronutrients such as vitamin C, carotene and vitamin E [68, 69].

As early as the 1970s, the low prevalence of CRC was studied in rural areas of many African countries, where it was observed that there was a higher intake of fiber and bulky stools with rapid evacuation [77].

The beneficial effect of dietary fiber to reduce the risk of CRC is based on the fact that it increases the volume of the stool and reduces the transit time, so that the exposure of the colorectal epithelium to carcinogens is reduced [78]. Although another mechanism of action involves the interaction of fiber with the intestinal microbiota. Soluble fiber reaches the colon undigested, where it is fermented by the anaerobic intestinal microbiota into short chain fatty acids, mainly acetate, butyrate and propionate [79]. Among its actions is the reduction of pro-inflammatory pathways [80], but also that of favoring the survival of normal colonocytes and apoptosis of neoplastic colonocytes, as shown by *in vitro* studies carried out with human intestinal cells [81].

Although dietary fiber has been inversely associated with the risk of CRC, that the source of the fiber is important should be noted. Thus, fiber from whole grains has been significantly related to reducing the risk of CRC [82] and adenomas [83], but not although not all studies show significant differences for fiber from fruits, vegetables and legumes. Even the amount of fiber from whole grains has been inversely associated with the incidence and mortality of CRC [82, 84].

Since living beings are a network of biological pathways, different pathways may appear that relate diet to CRC. Thus, there are foods that can predict levels of circulating C-peptide (indicator of insulin secretion) and inflammatory markers (such as C-reactive protein (CRP), Interleukin 6 (IL-6), Tumor necrosis factor (TNF) receptor 2) [85]. In a healthy diet with intake of whole grains the levels of peptide-c and inflammatory markers are low, but in an unhealthy diet pattern with high intake of meat, the levels of C-peptide

and pro-inflammatory markers increase and this increase is associated positively with CRC risk [86], suggesting that the effects of diet on CRC risk could be mediated, in part, by insulin levels and inflammation.

1.3. MECHANISMS/PATHOLOGY

1.3.1. INTESTINAL EPITELIUM

The small intestine and the colon are maintained for life thanks to progenitor cells with an extraordinary capacity for proliferation and differentiation, which gives the intestine a great capacity for self-renewal. Both the epithelium of the small intestine and that of the colon are structured in finger-shaped protrusions called villi that are designed to maximize the absorption surface. These villi are surrounded by epithelial invaginations called crypts, forming structural and functional units of the intestine, which are the place where progenitor cells are found [87].

The different cells that make up the intestinal epithelium are:

- Enterocyte, polarized cells that are responsible for absorption, are the majority cell type.
- Goblet cells, secrete mucin.
- Enteroendocrine cells, participate in the release of hormones.
- Plume cells, in charge of detecting the content of the lumen.
- Microfold cells act as portals for light antigens and are located in Peyer's patches.
- Paneth cells are located exclusively within the crypts, secrete bactericidal proteins and help protect intestinal stem cells.
- Intestinal stem cells, are located in the deepest area of the crypts and are responsible for the self-renewal of the intestinal epithelium.

All these cell types have a strict arrangement, stem cells are located at the bottom of the intestinal crypts, where they produce highly proliferative cells which differentiate as they move up the crypts and give rise to various types of mature epithelial cells that make up the intestinal lining [22]. After differentiation, the stem cells remain at the bottom of the crypts, while their progeny moves up the walls of the crypt, creating different levels of differentiation [88].

Most crypt cells have a short half-life, except for Tuft cells, neuroendocrine cells, and Paneth cells, which are specialized cells with a long half-life. Due to the short half-life of these cells, a large number of colon epithelial cells, on the order of 10^{14}, must be produced during the half-life of humans [24]. This great proliferation of colonocytes occurs from the epithelial stem cells of the colon, through a strongly regulated pathway, which allows to regulate proliferation to the physiological needs of the individual.

Stem cell maintenance is mainly carried out by Wnt signaling pathways [89]. The importance of the Wnt pathway in the maintenance, proliferation and differentiation of intestinal stem cells is based on the fact that the target genes Wnt/β-catenin show their maximum expression in cells at the base of the crypt [24]. The interest of the Wnt pathway focuses on the fact that high signaling in stem cells leads to uncontrolled proliferation and, this in turn, to tumor formation. In fact, most colon cancers are associated with some mutation that activates the Wnt pathway [22].

1.3.2. CARCINOGENIC MECHANISMS

CRC is a very heterogeneous disease that manifests itself in various clinical and molecular characteristics, in addition to sensitivity to treatments and its prognosis. Both genetic and environmental factors gradually modify the cells of the intestinal epithelium of the colon until they acquire the characteristics of cancer cells [90, 91].

The origin of most cases of CRC is in a stem cell or a stem cell-like cell that resides at the base of the crypts of the colon [92]. These epithelial cells of the colorectal mucosa are subjected to the loss of genetic/epigenetic stability, which is why mutations and alterations in the oncogenes and tumor suppressor genes accumulate, leading to the malignant transformation of the cells and their subsequent clonal expansion [25, 93, 94].

The current pattern of CRC formation suggests that most arise from a polyp, from an aberrant crypt. Therefore, they invariably arise from benign precursor polyps that show a progressive and staggered accumulation of genetic and epigenetic changes that are the main drivers of tumor development [95], these changes that induce the formation of glandular structures called adenocarcinomas [96], from which most of the CRCs will develop according to the adenoma-carcinoma sequence [9].

Regardless of the reason for the genetic or epigenetic changes, for CRC to develop, single mutations are not enough to trigger the carcinogenic process in the intestinal epithelium [96], but rather originate as a consequence of the accumulation of mutations in oncogenes, tumor suppressor genes and DNA repair genes, leading to loss of normal mechanisms of cell growth and differentiation.

The molecular changes associated with the tumor progression of CRC are mainly attributed to genomic instability by which the accumulation of somatic aberrations occurs, the three main routes of action are: control of gene expression by microsatelites, chromosomal instability (CIN) and DNA methylation, especially of the CpG island [1, 95].

Genomic instability is associated with 85% of CRC cases and was proposed by Fearon *et al* [25] such as the suppressive pathway or CIN pathway [97]. CIN is characterized by changes in the number of chromosomes [98], as well as by structural abnormalities and

loss of heterozygosity and amplifications, in addition to the inevitable mutations in oncogenes and tumor suppressor genes [24, 97]. Poor chromosomal segregation can lead to mutations in the APC gene that activate the Wnt signaling pathway, a key step in the initiation of 80% of CRCs [22]. When the TP53 gene is mutated, a cell cycle checkpoint is lost and uncontrolled cell proliferation occurs and drives the progression of carcinogenesis [25, 26].

On the other hand, microsatellite instability (MSI) pathway, also called the mutator pathway, is responsible for 15-20% of sporadic CRC [9, 97, 98]. It is characterized by the production of errors in the mismatched base repair (MMR) mechanisms. The MMR genes act in the S phase, repairing errors in the DNA produced by base mismatch or as a consequence of the slippage of DNA polymerase during the replication of highly repeated sequences [99]. Defects in these genes result in point mutations as well as insertions/deletions that change the reading frame and produce a premature stop codon, which codes for a non-functional protein [100]. Genes that contain repeating sequences within the coding region [101], such as APC, MLH1, transforming growth factor beta receptor 2 (TGF/BRII), and BCL2 Associated X (BAX) [102], have an increased risk of MMR. The MMR system is made up of seven repair genes: MLH1, MLH3, MSH2, MSH3, MSH6, PMS1 and PMS2 [103, 104]. This pathway produces DNA instability, generating somatic alterations in specific regions of microsatellites [24], and leads to CRC through the accumulation of mutations that occur at high speed and inactivate genes responsible for the base-pairing repair system [98]. The accumulation of pairing alterations occurs mainly in the microsatellite regions of DNA with repetitive nucleotide sequences [13]. MSI is detected by comparing the microsatellite copy number of tumor cells with that of normal cells; tumor cells generally have higher microsatellite copy numbers [97].

Epigenetic mechanisms can also play a role in the development of CRC, mainly via DNA methylation. Epigenetic modifications can involve methylation of cytosine residues in DNA and/or changes in chromatin structure that regulate gene expression [105]. DNA methylation in CpG dinucleotides is one of the epigenetic mechanisms involved in the regulation of gene expression in mammals and the methylation patterns are specific for each species and type of tissue. The machinery involved comprises different regulatory proteins including DNA methyltransferases, putative demethylases, methylated CpG-binding proteins, histone modifying enzymes, and chromatin remodeling complexes [106]. DNA methylation is of vital importance to maintain gene silencing in normal development, genomic imprinting and inactivation of the X chromosome, in contrast, alterations in it are implicated in some human diseases, especially those related to defects in development and the neoplastic process [106]. In this way, 15% of sporadic CRC is generated [9, 97]. Epigenetic alterations appear commonly in polyps and CRC, it seems that associated with genetic mutations promote carcinogenesis [27, 28]. DNA methylation is a modification of DNA that has been identified as the third route of carcinogenesis [9]. The CpG rich regions or CpG islands are the regions of DNA that are affected by methylation. A crucial determinant of repression is the density of methylation in CpG regions near the promoter, weak promoters are fully repressed by sparse methylation, if the density of methylation is further increased, even the enhanced promoter cannot prevail and repression remains complete [105]. Hypermethylation can cause transcriptional silencing [29] including tumor suppressor genes [9, 24]. Hypomethylation of repetitive genetic elements is associated with the activation of oncogenes and genomic instability [30]. Tumors produced by methylation are more frequent in women and the elderly, with preferential location on the right side of the colon and do not benefit from treatment with 5-fluorouracil (5-FU).

1.3.3. CARCINOGENIC PATHS

Most CRCs begin as a growth of the inner lining of the intestinal epithelium that projects over the mucosa, these structures called polyps are benign tumors. Over time (usually many years), some polyps turn into cancer. Adenomatous polyps, or adenomas, are important precursor lesions of CRC [107], although they do not always lead to cancer.

The carcinogenesis of CRC can occur by three known pathways: adenoma-carcinoma sequence, serrated pathway and inflammatory pathway. The most common is the adenoma-carcinoma sequence, a process by which CRC originates from an adenoma [10]. This model includes the gradual and staggered accumulation of genetic and epigenetic alterations that will lead to the transformation of adenoma into adenocarcinoma. The serrated pathway is an alternative carcinogenic pathway in which CRC develops from serrated adenomas [108, 109]. In this way, 20-30% of sporadic CRC cases develop [110]. The inflammatory pathway is the third suggested carcinogenic pathway for the development of CRC. Chronic inflammation increases the risk of CRC, in a study carried out with patients with inflammatory bowel disease it was found that the increase was up to 2.4 times greater than that of the general population [111]. In this path, the evolution of dysplasia to CRC occurs [112]. It is estimated that less than 2% of CRC cases originate through the inflammatory pathway [113].

Then, in addition to genetic alterations, the tumor microenvironment plays a critical role in the initiation and promotion of CRC, with dietary intake and the intestinal microbiota being the most dominant factors in the luminal microenvironment of the intestine. Therefore, it has been suggested that differences in diet and gut microbiota could be responsible for variations in the prevalence of CRC between two similar human populations.

2. GUT MICROBIOTA

2.1. HEALTHY MICROBIOTA

The microbiota encompasses enormous biodiversity and plays an important role in ecosystems by supporting all living things, including humans. The human microbiota is composed of different populations of bacteria that reside in the epithelial barriers of different organs of the host with which they have a mutualistic relationship. Knowledge about the human microbiota is becoming more extensive, new taxa are being discovered in different anatomical sites and also their benefits or damages on human health, since the microbiota is an active ecosystem that interacts with the host. In 2018 the number of cultured bacterial species associated with humans was 2776, in 2020 the number increased to 3253, an increase of 17% in less than 2 years [114].

The importance of the intestinal microbiota on the health of the host has been known for more than a century [105], intensifying this interest in recent decades. The intestinal microbiota carries out different fundamental functions directly related to food, such as the production of essential vitamins. But it also has health-related functions such as the production of important metabolites, prevention of infestation by pathogens and the control of bacterial groups to avoid the increase of toxin-producing bacteria [115], in addition to intervening in the activation of the immune system [116].

Intestinal microbiota is a very wide term, since the diversity of bacterial groups and their quantity varies throughout the digestive tract, increasing from the stomach to the colon, where the densest and metabolically active bacterial community is found [117]. The digestive tract is home to between 10^{13} and 10^{14} microorganisms, a number that is almost 100 times the number of cells that make up the human body [118, 119]. Of the large number of existing intestinal microbes, the highly predominant domain is that of bacteria,

the number of species estimated is 500-1000 [118]. Of which 90% belong to the bacterial phyla Bacteroidetes and Firmicutes, and a lower presence of Verrucomicrobia and Actinobacteria. Although the phylum Firmicutes has more than 200 genera in the intestinal microbiota, *Clostridium*, *Lactobacillus*, *Enterococcus*, *Bacillus* and *Ruminococcus* stand out. From the phylum Actinobacteria, the genus *Bifidobacterium* [120].

The intestinal microbiota has been highly studied by sequencing the 16S ribosomal DNA of fecal samples. With this technique it has been observed that there is a great individual variation, the bacterial profile of each individual being different, these variations are greater as age advances [118].

However, when comparing the microbiota of different healthy individuals, similarities are observed that are relatively stable over time [118]. After birth, in a period that can last up to 3 years, an early bacterial composition is established that remains stable for years. Although there are studies that show that gut microbiota patterns can be transmitted from generation to generation [119]. Despite the great variability of the intestinal microbiota due to genetic influence [121] and the environmental factors that affect it (diet, chemical substances, drugs and antibiotics), a relatively stable composition of the intestinal microbiota has been established for healthy adults [122]. But the microbiota reacts to the dietary and health conditions of the host and to the environment of the intestine, which is conditioned by the epigenetics of the host, which in turn, responds to the environmental conditions in which it develops host [118].

Currently, multiple diseases, both intestinal and non-intestinal, have been associated with the intestinal microbiota, among which are several types of cancer with characteristic microbiome patterns and dysbiosis [123].

2.2. DYSBIOSIS IN COLORECTAL CANCER

Currently, it is known that the intestinal microbiota participates in physiological and pathophysiological processes that are related to various diseases, such as type II diabetes, obesity, atherosclerosis, inflammatory bowel disease or gastrointestinal cancer [119].

As early as the 1960s a study in rodents was carried out which demonstrated the carcinogenic effect of cycasin in conventional rats, but without the development of cancer in germ-free rats [124]. Which put on alert the relationship of the intestinal microbiota in the development of cancer. A subsequent study in which rodents were treated with another carcinogenic molecule, 1,2-dimethylhydrazine, identified the genera *Bacteroides*, *Clostridium*, *Enterococcus*, and *Escherichia* as possible promoters of colorectal carcinogenesis of carcinogen-induced aberrant crypt foci [125].

It has been established that there are significant changes in the composition of the intestinal microbiota when comparing the microbiota of healthy individuals with that of patients with CRC [119]. Recent studies have shown that in the case of CRC patients, bacterial diversity and richness is lower [126, 127]. With a lower abundance of potentially protective taxa (such as *Roseburia*) and a higher abundance of taxa considered procarcinogens (such as *Bacteroides*, *Fusobacterium*, *Escherichia* or *Porphyromonas*) [128].

This change in the composition of the intestinal microbiota that favors the enrichment of pro-inflammatory opportunistic pathogens and the decrease of butyrate-producing bacteria lead to an imbalance in intestinal homeostasis or dysbiosis plays an important role in the appearance and development of CRC [129, 130] since the intestinal microbiota creates a favorable tumor microenvironment [120, 128]. The fact that the intestinal microbiota is one of the important factors associated with CRC is currently indisputable,

identifying bacteria closely related to the disease [120]. This is the case of *Fusobacterium* and *Streptococcus* that activate metabolic cascades that are associated with an increased risk of CRC [119]. The association of *Fusobacterium* with genetic mutations in CRChas even been confirmed [131]. CpG island methylation phenotype status, MSI status, and wild-type p53 from tumor tissue are associated with increased abundance of Fusobacterium [119]. And some studies have even proposed the possible use of the abundance and detection rate of certain bacterial groups, as is the case of species belonging to *Fusobacterium*, *Bacteroides* and *Methanobacteriales*, which could be useful for the early detection of CRC [129]. Animal studies have shown the colorectal carcinogenic action of *Fusobacterium nucleatum*, *Escherichia coli*, *Bacteroides fragillis* [130], as well as *Enterococcus faecalis* and *Streptococcus gallolyticus*, which are individually associated with the development of CRC [128]. Thanks to metagenomics, it has been possible to carry out studies in humans in which new bacterial genera related to CRC have been identified, such as *Parvimonas*, *Peptostreptococcus*, *Phorphyromonas* and *Prevotella*, present in greater abundance in stool samples and tumor tissue of patients with CRC [128].

Bacterial groups such as *Bacteroides fragilis*, *Fusobacterium nucleatum*, *Enterococcaceae* or *Campylobacter*, *Peptostreptococus*, *Enterococus faecalis*, *Escherichia coli*, *Shigella*, and *Stolyreptococus* were significant increases, while *Faecalibacterium*, *Blautia*, *Clostridium*, *Bifidobacterium* and *Roseburia* were significantly decreased [132]. In another studies have been also described a decrease in the abundances of *Clostridium butyicum* [133] belonging to a family of Gram-positive fiber-fermenting bacteria [134] and *Streptococcus thermophilus*, both probiotic bacterial species used to prevent antibiotic-associated diarrhea [128].

2.3. CHANGES IN THE GUT MICROBIOTA IN COLORECTAL CANCER

The gut microbiota is partly responsible for the origin, development, and evolution of CRC. These microorganisms create a microenvironment conducive to carcinogenesis, but it is a changing environment that evolves together with CRC, so bacterial populations also tend to change. In 2012, Tjalsma *et al*., proposed a driver-passenger bacterial model through which it was intended to explain the participation of bacterial populations and their changes during the development of CRC [135]. This model may contribute to the genetic paradigm of the adenoma-carcinoma sequence.

The process begins when the colon mucosa of the individual at risk of CRC is colonized by pathogenic driver bacteria, which are intestinal bacteria with pro-inflammatory potential and procarcinogenic characteristics (*Bacteroides fragilis* and *Escherichia coli* in particular) [136]. Driver bacteria produce genotoxins and induce inflammation that lead to damage to the DNA of epithelial cells, which leads to the initiation of early mutations of the adenoma-carcinoma sequence [124, 137] and to modify the tumor environment whose changes will favor that driver bacteria are gradually replaced by opportunistic bacteria with competitive advantages in the new environment [138]. These opportunistic bacteria or passenger bacteria (*Fusobacterium nucleatum* and *Streptococcus gallolyticus* in particular) have the ability to promote further development of CRC [139].

Therefore, there are no bacteria that affect the appearance and development of CRC by themselves, but it is a process in which the bacterial community participates jointly or sequentially throughout the carcinogenesis process [139, 140].

A look at the large bacterial taxa of the intestinal microbiota shows us how in the initial stage of the tumor, when comparing a model group of mice with CRC induced by 1,2-

Dimethylhydrazine with a control group, there are significant differences in Bacteroidetes and a lower relative abundance of Firmicutes and Proteobacteria [141].

Among the driving bacteria we find *Bacteroides fragilis* and genotoxic *Escherichia coli*, which produce genotoxic substances that cause DNA damage [128]. Colibactin is produced by *E. coli*, this genotoxin is capable of inducing single-stranded DNA breaks, which results in increased mutations in affected cells [142]. *Shigella* spp is a potentially pathogenic enterobacteria and, as such, it tends to appear rarely in healthy individuals, however it is overrepresented in samples from patients with non-malignant adenomas [136, 143]. Other species with high initial representation and that decrease when the tumor develops are *Clostridium lactatifermentans* and *Bacteroides dorei*, with a proinflammatory character and induce hyperproliferation. They are also from the group of enterotoxigenic inflammatory bacteria *Bacteroides fragilis* or the Enterobacteriaceae family, which can also cause dysplasia, hyperplasia and genotoxins [141].

Unlike driver bacteria, passenger bacteria are not good colonizers of a healthy colon, but the tumor microenvironment gives them a competitive advantage [96] since there is a breakdown of the colonic barrier, increased tumor vascularization and infiltration of immune cells that are correlated with local physiological and metabolic changes [144]. *Streptococcus gallolyticus* subsp. gallolyticus is more prevalent in patients with colon adenomas [145-147] which is due to the fact that these bacteria use exposed collagen fibers of the basement membrane of a tumor environment to access the affected tissue [148].

Fusobacterium spp is overrepresented in tumor samples [145, 146], it is considered a transient bacterium [135] with inflammatory capacity [149] and invade tissues [150], thus contributing to the change of the tumor environment [151, 152]. The higher abundance

of *Fusobacterium nucleatum* in the early phases of dysbiosis in patients with adenomas was related to a higher expression of pro-inflammatory cytokines [153, 154].

2.4. INFLAMMATION AND REGULATION OF THE IMMUNE SYSTEM BY GUT MICROBIOTA IN CRC

Inflammation has been proposed as a promoter of carcinogenesis, and it plays an important role in the initiation and evolution of CRC. Chronic inflammation has been established as a mark of the appearance of cancer [91] due to the greater risk that patients with chronic inflammatory disease have to develop it [155]. It is estimated that up to 20% of cancer cases are preceded by chronic inflammatory processes [156].

In view of some studies that have been carried out to date, it has been established that there is an association between colorectal adenoma and circulating levels of CRP, which is a marker of systemic inflammation although it is not specific [157]. High levels of pro-inflammatory cytokines have also been observed in adenomatous tissues, such as tumor necrosis factor alpha (TNF-α) or interleukin-6 (IL-6), both of which participate in the processes of cell growth, differentiation and apoptosis [158, 159].

Enterotoxigenic *Bacteroides fragilis* (ETBF) is also enriched in CRC patients and produces inflammation through the action of interleukin 17 (IL-17), signal transducer and activator of transcription 3 (STAT3) and nuclear factor-kappa B (NF-kB), all activated by its virulence factor [128]. ETBF is capable of colonizing Adenomatous polyposis coli/multiple intestinal neoplasia (APC/Min+) mice and activating the STAT3 pathway [113, 140] and stimulates the cells of the intestinal epithelium of the colon to produce particles necessary for the recruitment of helper T lymphocytes 17 and the activation of IL-17, favoring the growth and survival of tumor cells [160]. ETBF also promotes

inflammation by altering the intestinal barrier, as it can induce the degradation of E-cadherin in the colon epithelium [161].

A study carried out in germ-free mice that were fed by gavage with feces from patients with CRC, showed an increase in inflammatory genetic markers and histological inflammation since the microorganisms induced the production of chemotactic factors in the gut, such as the C-X-C motif chemokines ligands 9 and 10 (CXCL9 and CXCL10), for cytotoxic T lymphocytes and type 1 helper T lymphocytes, and as C-C motif chemokines ligands 17 and 20 (CCL17 and CCL20), for IL-17 producing helper T cells [160]. Cancer cells are also known to release inflammatory cytokines and chemokines that attract pro-inflammatory helper T cells. Thus, a tumorigenic microenvironment is created in which growth and angiogenic factors and tissue remodeling enzymes abound, and the antitumor response of T cells is suppressed [162].

Moreover, currently, a practically indivisible association between the gut microbiota, inflammation and the immune system is assumed in CRC. Gut bacteria induce an immune response that can cause ongoing low-grade inflammation, which can promote tumorigenesis. Although it has been seen that inflammation cannot induce the tumor without the presence of the microbiota or its derived compounds [163]. Inflammation of the colon could be a key factor in the development of CRC and is extremely associated with bacterial dysbiosis and increased intestinal permeability [164]. Changes in the balance of commensal bacteria can lead to increased intestinal permeability, bacterial translocation, and activation of the innate and adaptive immune system that stimulate chronic inflammation [165].

Mice studies have shown that there is a relationship between the appearance of polyps and defects in the intestinal barrier of the colon, bacterial invasion and a greater

expression of inflammatory factors such as TNF-α, IL-1, IL-17 and C-X-C motif chemokines ligand 2 (CXCL2). Alterations in the intestinal mucosa barrier are key for the microorganisms to induce local inflammation [166]. As a result of this inflammation, cytokines and chemokines are secreted that act as growth factors and angiogenesis is stimulated [162].

When the barrier of the intestinal mucosa is altered, intestinal bacteria and their degradation products (such as lipopolysaccharides of the outer membranes of some bacterial groups) can penetrate into the tumorigenic tissue where the host recognizes them through receptors, such as Toll-like receptors (TLR), which control the inflammatory response to microorganims associated molecular patterns (such as lipopolysaccharides) [167]. During carcinogenesis, tumor cells produce pro-inflammatory cytokines and chemokines that attract immature myeloid cells or pro-inflammatory helper T cells [168]. Invasive commensal bacteria of tumor tissue and their components activate the TLR receptors of the myeloid cells present, after activation of myeloid differentiation factor 88 (MyD88) through the production of inflammatory cytokines, the most notable of which are IL-23, which in turn activates IL-17, IL-6 and IL-22 [167, 169, 170], thus activating the NF-kB and the STAT3 signaling pathway, promoting the proliferation of tumor cells [171, 172]. The activation of NF-kB, STAT3, the production of reactive oxygen species (ROS) and the related oxidative stress and the damage produced in the DNA, favor an abnormal cell proliferation that leads to the development of colorectal adenomas and CRC [173, 174].

More specifically, some bacterial species and their relationship with CRC inflammation have been identified. When *Fusobacterium nucleatum* is enriched in cases of human CRC, interleukin IL-17A has an increase in its expression [175]. It has also been seen in mouse casts that *F. nucleatum* generates a pro-inflammatory environment that allows the

activation of the NF-kB pathway and the infiltration of the tissue by immune cells, allowing the progression of colorectal neoplasia in APC/Min mouse models (a common mouse model of CRC) [140, 149]. *F. nucleatum* can activate TLR4 signaling to promote tumor development in mice [176].

Peptostreptococcus anaerobius also favors tumorigenesis by participating in the formation of a pro-inflammatory environment through the induction of inflammatory cytokines, recruitment of immune cells in tumor tissue, especially immunosuppressive myeloid-derived suppressor cells, tumor-associated macrophages, and granulocytic tumor-associated neutrophils, to promote tumor progression [177]. It has also been seen in mice that this CRC-enriched bacterium can promote tumorigenesis by activating the TLR2 and/or TLR4 pathways [178].

2.5. PRODUCTION OF GENOTOXINS IN COLORECTAL CANCER

Microorganisms also affect the carcinogenic process through the production of toxins that damage DNA, which is known as genotoxins. Among toxins of this type is the cytolethal strain toxin (CDT), which is produced by enteric pathogens such as *Escherichia* and *Campylobacter* spp and is capable of inducing double-stranded DNA breakage through its deoxyribonuclease activity [128, 140]. The carcinogenic process has been shown to be attenuated in mouse models that are deficient in CDT [179].

Colibactin is produced by the Enterobacteriaceae family, being known as the *Escherichia coli*. Colibactin causes DNA cross-linking and double-stranded DNA breaks [180]. In addition to DNA damage in eukaryotic cells and chromosomal instability, colibactin induces apoptosis of immune cells [130]. The participation of this toxin in carcinogenesis has been demonstrated when the use of small molecule inhibitors targeting colibactin reduced the tumor burden of a mouse model [181].

Bacteroides fragilis toxin is a zinc-dependent metalloprotease that induces the breakdown of E-cadhenin (tumor suppressor protein), increasing the signaling of the Wnt/β-catenin pathway, as a result, an increase in the permeability of the intestinal barrere, the expression of the MYC oncogene and the greater proliferation of the tumor cells of the colon [12]. This toxin also triggers the NF-kB signaling pathway that contributes to inflammation through cytokine production by mucosal epithelial cells [182]. In addition, the toxin from *B. fragilis* has been studied *in vitro* and has been associated with DNA damage and genomic instability [128]. Furthermore, it has been seen that *B. fragilis* induces the action of the catabolic enzyme polyamine spermine oxidase, which converts spermine into spermidine at the same time as it generates H_2O_2, this ROS form causes DNA damage [96].

By the way*, Enterococcus faecalis* produces reactive oxygen species that damage DNA and cause genomic instability, as seen *in vitro* in human cells [96, 128]. In colon epithelial cell cultures, *E. faecalis* activates DNA damage pathways, causes cell cycle arrest in G2, and induces erroneous chromosome segregation. The carcinogenic effect has also been demonstrated *in vivo* in a study carried out with IL-10 deficient gnotobiotic mice that were colonized with *E. faecalis* [96]. The AvrA protein from *Salmonella* influences eukaryotic cell pathways by regulating ubiquitination and acetylation [183], inducing β-catenin signaling and enhances colonic tumorigenesis by activating the STAT3 pathway, as seen in mouse models with CRC [140, 184].

Finally*, Fusobacterium nucleatum* has an exclusive membrane adhesin called FadA through which it can bind to E-cadherin on the surface of colon cells, when this occurs the activation of the Wnt/β-catenin signaling pathway occurs and the consequent inflammatory response and oncogenesis [140, 185]. Another *F. nucleatum* surface adhesin, Fap2, can bind to the inhibitory immune receptor TIGIT (T-cell immune receptor

with immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domains) and alter the function of natural killer and tumor cells [186].

2. 6. MEDIATORS OF COLORECTAL CANCER CARCINOGENESIS
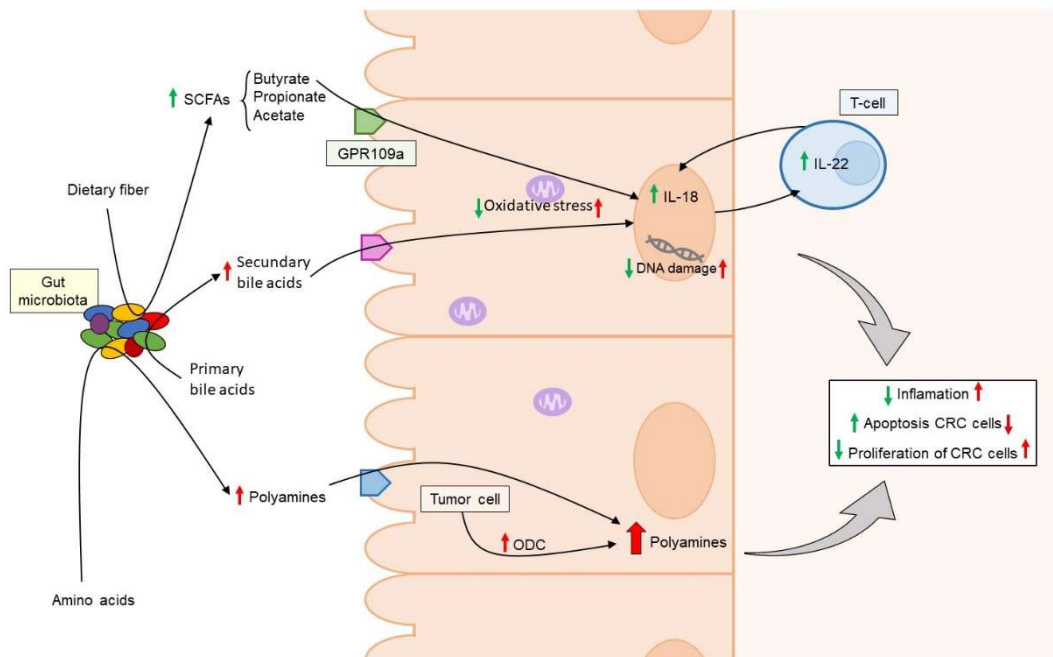
Figure 3. Mechanisms of action of short-chain fatty acids, bile acids and polyamines (microbiota-derived metabolites) in the inflammation, apoptosis and cellular proliferation of colon cancer cells. The green arrows indicate the mechanisms that favor a healthy state. The red arrows indicate the mechanisms that increase cancer risk. SCFAs: short chain fatty acids; ODC: ornitina descarboxilasa; GPR109a: G-protein-coupled receptors; IL: interleukin**.**

The variety of bacteria that are part of the intestinal microbiota contribute to the physiological and health status of the host through biochemical pathways related to their versatile metabolic genes [187]. The gut microbiota can influence colorectal carcinogenesis through a variety of mechanisms, including metabolites derived from the anaerobic fermentation of undigested exogenous dietary compounds. Today there are

multiple studies that have shown that metabolites such as short-chain fatty acids contribute by protecting the host against CRC, in contrast to other metabolites such as secondary bile acids or polyamines [187] (**Figure 3**).

2.6.1. SHORT CHAIN FATTY ACIDS

Short chain fatty acids (SCFAs) are molecules derived from microbial metabolism from components of the diet. These metabolites (mainly butyrate, propionate and acetate) are very important in the digestive tract where they promote the health of the host [140]. SCFAs are produced through the anaerobic fermentation of some bacterial groups, the major producers of butyrate belong to Firmicutes [188], those of propionate to Bacteroides, Firmicutes and *Propionibacterium* [188, 189] and those for acetate are *Acetobacterium*, *Clostridium aceticum* and *Propionibacterium* [188, 190]. SCFAs influence the control of gene expression through epigenetics, mediate the inflammatory response, help maintain the intestinal barrier and protect against oxidative stress [191].

The rate of production of SCFAs and the amount present depends on the bacterial groups existing in the colon and their abundance, the source of the substrate and the intestinal transit time [192]. The increased risk of CRC is associated with a bacterial dysbiosis that leads to a reduction in the production of SCFAs [192, 193] especially of butyrate which is the most potent as a protector against cancer [140]. The reduction in pH of colonic stools produced by the presence of SCFAs (especially butyrate) inhibits the proliferation of pathogens and DNA damage, in addition, it prevents the proliferation of tumor cells and improves apoptosis [140], this pH is elevated in CRC patients compared to healthy individuals, which could be due to a lower amount of SCFAs [194].

The main source of energy for colonocytes are SCFAs, however, CRC-altered colonocytes obtain energy mainly from aerobic glycolysis [194]. Despite this, CRCcells are prone to uptake SCFAs [195], which denotes the importance of these molecules in cell homeostasis. Normally, intestinal bacteria exist in a mutually beneficial symbiotic relationship with the human colon, providing the necessary amount of SCFAs to maintain colon homeostasis [194].

SCFAs (mainly butyrate) participate in the maintenance of the integrity of the intestinal barrier through the regulation of tight junction proteins [187], in particular claudin-1 [191]. They also produce increased release of the anti-inflammatory cytokine IL-10 which protects against cancer formation. In a recent study, the low levels of IL-10 in patients with obesity and CRC were associated with a lower abundance of butyrate-producing bacteria such as *Blautia* and *Faecalibacterium prausnitzii*, when compared with healthy individuals [196].

SCFAs are also capable of inducing the production of antimicrobial peptides and regulate the functions of regulatory T cells, thus participating in the host's immune response [191]. It has been seen in animal models that butyrate and propionate can influence the regulatory T cells of the colon [140] producing an important anti-inflammatory effect [128]. SCFAs participate in this process by interacting with certain receptors on the membrane of host cells, such as G-protein coupled receptor 43 (GPR43) for acetate, G-protein coupled receptor 41 (GPR41) for propionate, or G-protein coupled receptor 109 (GPR109) for butyrate [140, 187]. Specifically, the union of butyrate with the GPR109 receptor activates the production of IL-18, which in turn induces the release of IL-22 by regulatory T cells, which will stimulate the repair of the epithelial mucosa tissues and the decreased inflammation [197]. In studies with mice, the absence of IL-18 has been related

to bacterial dysbiosis and alterations in the immune response, mucosal repair and homeostasis [198], which favors tumorigenesis.

In vivo observations have shown that the loss of SCFA receptors can contribute to colon tumorigenesis due to the change in the intestinal microbiome, the alteration in the integrity of the intestinal barrier, the over-activation of dendritic cells and the inactivation of CD8+ T cells [187]. Butyrate is particularly credited with the ability to induce the expansion of regulatory T cells, regulating the local immune response and suppressing colon inflammation and, therefore, tumorigenesis [199].

2.6.2. BILE ACIDS

Bile acids are important bacterial metabolites that participate in the digestion of food, in the health of the host and, therefore, their imbalance or malfunction also participate in disease processes. Through the breakdown of cholesterol, primary bile acids such as cholic acid (CA) and chenodeoxycholic acid (CDCA) are synthesized in the liver, which are transformed by intestinal bacteria through the 7α/β-dehydroxylation pathway into secondary bile acids, such as deoxycholic acid (DCA) and litholic acid (LCA) [128, 187, 200].

Bile acids, especially secondary bile acids, are potent carcinogens, high physiological levels of them for a long time can induce CRC [200]. The imbalance in the levels of bile acids induces inflammation and damages the colon epithelium, because they are related to the production of ROS [201]. Moreover, the bile acid, also favoring genomic instability and to inhibit cell apoptosis [201]. *In vitro* and *in vivo* studies using colon biopsies, it was seen how secondary bile acids stimulated the proliferation of colonic epithelial cells, DNA fragmentation and oxidative damage, which resulted in an increase in the expression of cyclin D1, a molecule that participates in the cell cycle through the Wnt/β-catenin

signaling pathway and to degrade the tumor-suppressor p53, while promoting resistance to apoptosis [202]. Mutations affecting the Wnt/β-catenin signaling pathway [203] have been detected in more than 90% of CRC cases, and in a study with rodents administered DCA, the levels of β-catenin and cyclin D1 increased, which favored low-grade intestinal inflammation, the alteration of the intestinal barrier and, as a consequence the, tumorigenesis [202].

Among the receptors that bile acids interact with are the Farnesoid X receptor (FXR) and the G protein-coupled receptor (GPBAR1 or TGR5) [204]. Bile acids bind and activate these receptors, thus maintaining energy and metabolic homeostasis [201]. Recently a relationship between FXR and the Wnt/β-catenin signaling pathway has been found [203]. Silencing FXR expression in APC/Min+ mice showed tumorogenic progression and accelerated mortality through activation of the Wnt/β-catenin signaling pathway [201]. Whereas FXR overexpression in CRC cells, both *in vitro* and *in vivo*, suppressed tumor progression [203].

The secondary bile acid LCA, have been associated not only with the promotion of colorectal tumorigenesis, but also with metastasis and poor prognosis of the patient through stimulation of interleukin 8 (IL-8) or C-X-C motif chemokine ligand 8 (CXCL8) [205]. CXCL8 is a pro-inflammatory interleukin that regulates pathological angiogenesis, tumor growth, and metastasis, and their levels are elevated in CRC patients [187, 205]. The transcription factors NF-kB and activator protein (AP-1) have long been known to be involved in the expression of CXCL8 [206]. An *in vitro* study showed that LCA-induced upregulation of CXCL8 can be eliminated with metformin, which is able to suppresses the activity of NF-kB and Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (an important enzyme in ROS production) [207]. More recently, Nguyen *et al*. demonstrated that LCA induced CXCL8 expression in CRC HCT116 cells

by activating the extracellularly regulated kinases 1 and 2 (ERK1 and ERK2) and suppressing STAT3 [205].

Secondary bile acids can induce the mitogen-activated protein kinase (MAPK) pathway to stimulate the progression of CRC. DCA was shown as a possible inducer of tumorigenesis through the positive regulation of MAPK (mediated by calcium signaling) [187], the downregulation of apoptosis, the inhibition of tumor suppressors of the Erk1/2 and caspase-3 signaling pathways, the increase of ROS levels and the DNA damage [208]. In a study carried out in an African-American population, it was seen that lower levels of bile acids and DCA-producing bacteria as a consequence of the modification of the diet (less fat and more fiber), was correlated with the alteration of the associated mucosal markers at the risk of CRC, with cell proliferation, and also with the infiltration of immune cells (CD3+ and CD68+) in colonic tissue [209].

2.6.3. POLYAMINES

Polymianes are small polycationic aliphatic amines derived from L-arginine metabolism. Polyamines are produced by all eukaryotic cells although the main source is their ingested food and the microbiota [210].

Due to the high rate of turnover of the intestine there is a continuous cell proliferation that requires the presence of polyamines to facilitate the process of protein translation. It has been seen in rodents that in the proliferation zones of the crypts of the small intestine and colon, the concentration of polyamines is higher than in the rest of the gastrointestinal tract [211].

The relationship of polyamines with cancer has been known since the end of the 20th century, elevated levels of urinary polyamines were observed in patients with some types

of tumors as well as higher concentration of polyamines were found in cancerous tissues compared to healthy tissues [212]. Therefore, the metabolism of polyamines has been found deregulated in several types of cancer, including the CRC [196].

The involvement of polyamines in carcinogenesis may be linked to the adenomatous polyposis coli gene APC/c-MYC pathway. The APC protein is an important tumor suppressor whose gene is mutated in most CRCs. APC binds to β-catenin and activates the Wnt signaling pathway. In CRC tumor tissues, the defective APC causes that β-catenin to accumulate in the cell nucleus, activating a series of reactions that end the transduction of the c-MYC and KRAS genes, which activate the expression of genes such as ornithine decarboxylase (ODC) [213].

ODC is the key enzyme in the biosynthesis of polyamines and has been shown to have greater expression in tumor tissues compared to underlying tissues in the case of CRC [214, 215], which may indicate the possible relationship between polyamines. and cancer. Several *in vitro* studies have shown that a treatment with an ODC inhibitor, α-Difluoromethylornithine (DFMO), is effective in reducing the levels of putrescine and spermidine polyamines, but not always those of spermine effect that is reversible with exogenous polyamines [216]. Since endogenously synthesized polyamines can be replaced by exogenous polyamines, for DFMO treatment for colorectal adenoma to be promising, it should be used in combination with steroidal anti-inflammatory drugs [217]. Since the CRC tumor cells are in contact with intestinal bacteria capable of producing high levels of polyamines, studies with mice showed that the combination of DFMO with antibiotics improved the cytostatic effect of the treatment [218].

The catabolic enzyme polyamine spermine oxidase (SMO) is another enzyme of polyamine metabolism that can influence tumorigenesis through the production of ROS.

SMO intervenes in the conversion of spermine into spermidine, generating H_2O_2. It has been seen that in *Helicobacter pylori* and *Bacteroides fragilis* infections the main source of ROS is associated with SMO [96]. These bacteria promptly induce SMO, which generates ROS and causes DNA damage.

In addition, acetylated forms of polyamines have been shown to be more specific for cancer, it has been seen that levels of N1, N12-diacetylspermine are increased in CRC and dysplasic colorectal lesions [219]. In a study CRC patient, when tumor tissues and adjacen healthy tissues were compared, the appearance of a bacterial biofilm was described. This biofilm was able to affect both tumor tissue and healthy colon tissue and it was associated with a greater proliferation of colonocytes and an improvement in the metabolism of polyamines [220]. Metabolomic analyzes showed an association between the increase levels of N1, N12-diacetylspermine and the bacterial biofilm present in the intestine of the CRC patients, regardless of whether it was cancerous or healthy tissue [221]. Moreover, how the polyamines from the lumen of the intestine can be carried into the host cells, human HT-29 colon cancer cells were exposed to N1, N12-diacetylspermine and this polyamine can be detected intracellularly 24 hours after the dosage [211].

2.6.4. TRIMETHYLAMINE/TRIMETHYLAMINE N-OXIDE

Trimethylamine N-oxide (TMAO) is an oxidized metabolite of trimethylamine (TMA) that can be produced by the intestinal microbiota [222] and its level is related to risk of suffering from cancer, including CRC [223], with a positive correlation between TMAO levels and the increased risk of carcinogenesis. TMAO is considered as a potential indicator of CRC [187].

Precursor molecules such as choline or L-carnitine from the diet are converted to TMA by TMA-producing intestinal bacteria. TMA is absorbed by the intestinal mucosa and travels to the liver where it is metabolized into TMAO through oxidation catalyzed by the Flavin Monooxygenase (FMO) family, FMO-3 [223] and FMO-1 are the main isoforms, although its operation depends on genetic variability [224].

The importance of the role of the intestinal microbiota in the production of TMAO was described in a study in which participants were treated with antibiotics to eliminate their intestinal microbiota and were given carnitine. In these patients the levels of TMAO were measured in plasma and urine and they were significantly reduced when compared to untreated controls [223]. The relationship of the intestinal microbiota with the formation of TMA was also seen in another study, in which the levels of TMA were increased after an *in vitro* incubation of mouse intestinal medium supplemented with choline. In choline metabolism a carbon-nitrogen bond is cleaved, which can be carried out by intestinal bacteria [224]. Romano *et al*. related the increase in TMA with bacteria of the phyla Firmicutes and Proteobacteria, but not with Bacteroidetes, despite being one of the most abundant phyla in the human intestine [225].

Although there are some studies in which there is no relationship between TMAO and the risk of CRC, there are more and more studies that relate plasma levels of TMAO with the risk and development of CRC [223, 226] or with an unfavorable prognosis in CRC patients [226]. It has even been determined that the possible routes of action of TMAO in the pathogenesis of cancer (not only CRC) include cell progression and the Wnt signaling pathway [223].

TMAO has a pro-inflammatory role through the expression of genes that include IL-6 and chemokine ligands, as has been seen in cases of *Helicobacter pylori* infection. Or the

positive correlation between serum levels of TMAO and pro-inflammatory mediators, such as TNF-α and IL-6, in diabetic patients with chronic kidney disease. It is shown that this pro-inflammatory function does participate in the progression of cancer since TNF-α and IL-6 are involved in tumorigenesis through chronic inflammation [223].

Inflammation-activated oxidative stress linked to TMAO is also associated with cancer. Circulating TMAO levels have been shown to induce the production of ROS related to oxidative stress [223], which was also demonstrated in an *in vitro* assay [227]. Although there could also be links between TMAO and CRC, DNA damage and alteration of protein folding could also play a role in CRC development [134].

A recent study such as the recent study in which it has been proved that obese patients with CRC had higher levels of TMAO than non-obese patients with CRC or individuals healthy [228].

Although the course of action is still being elucidated, there are many studies proposing TMAO as a marker linked to CRC risk [222, 226, 229].

3. OBESITY, MICROBIOTA, COLORECTAL CANCER

There are more and more studies that show the relationship between obesity and CRC, both diseases have been studied separately and the role that the intestinal microbiota plays in each of them has been seen. We currently have evidence indicating the importance of the pathogenic potential of the gut microbiota on obesity and associated metabolic disorders, including cancer [201] (**Figure 4**).

The BMI according to the WHO gives us the definition of overweight, considering overweight a BMI of 25-29.9 kg/m^2 and obesity if the BMI is equal to or greater than 30 kg/m^2. Overweight and obesity alone are a major health risk and ranks fifth in overall risk

of death globally [230]. It must be taken into account that BMI is a crude measure, so other parameters such as waist circumference or adipokines would provide information to better understand the relationship of obesity, inflammation, and the microbiota with CRC [231]. As demonstrated for cases of lung cancer where a high BMI suggested a lower risk of cancer, however, in the same patients, the waist-hip ratio or the circumference of the waist were associated with a higher risk of lung cancer [231].
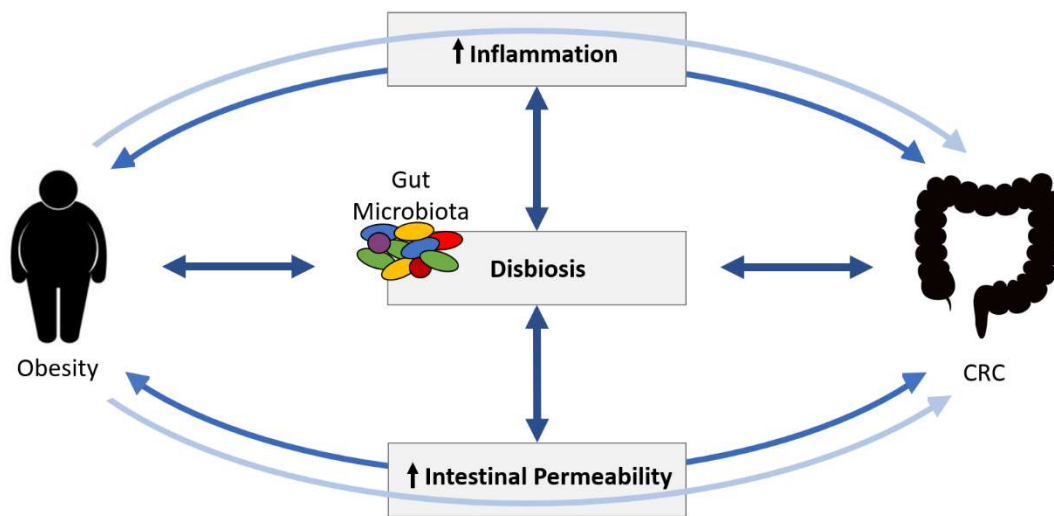
Figure 4. Influence relationship between obesity, the gut microbiota and colorectal cancer through intestinal dysbiosis and the processes of inflammation and intestinal permeability. CRC: colorectal cancer.

For many types of solid cancers, especially CRC, obesity is considered an important risk factor [232]. 14% of CRC cases are attributed to overweight and obesity [223]. Previous studies have determined an increased CRC risk of 7-60% in obese people compared to people with normal weight [232].

There is currently much speculation about the mechanisms that link obesity and CRC. Although in a meta-analysis it was determined that the composition of the intestinal

microbiota shows a weak effect in determining the risk of CRC in obese individuals compared to individuals with normal weight [231], the intestinal microbiota has been nominated as a link between both diseases. A recent study has found a similar microbiological profile in CRC patients and obese individuals [233]. Dysbiosis is generally observed in obese people which can lead to the proliferation of opportunistic pathogens and driver bacteria that will induce low-grade inflammation in the intestinal mucosa, which, when persisting over time, increases the probability and conditions that will favor the development of cancer.

In a study comparing the composition of the intestinal microbiota of patients with CRC, obese patients and healthy controls, it was observed that the species *Hafnia alvei* (Proteobacteria phyla) and *Akkermansia muciniphila* (Verrucomicrobia phyla) increased in the groups with CRC and with obesity [233]. These microorganisms are mucin degraders, which can lead to inflammation and tissue damage.

On occasions it has been suggested that it is diet and not obesity that induces the pro-inflammatory gut microbiota capable of increasing the risk of CRC [223]. Lipopolysaccharide (LPS) is an endotoxin produced in the intestine by gram-negative bacteria and is attributed with the production of low-grade chronic inflammation. Obesity induced by a high-fat diet favors an increase in LPS-producing bacteria and, therefore, circulating LPS levels. Colitis and associated CRC worsen with increased systemic endotoxemia and permeability of the intestinal barrier [201].

Germ-free animal models have been a great resource for studying the relationship of the gut microbiota with obesity and CRC. When dietary-induced obesity models were used, it was found that germ-free animals ingested more food and gained less weight than control animals with microbiota. They have also been seen as models of colon cancer,

spontaneous and induced by carcinogens, did not develop tumors in most cases in germ-free animals [201]. The specific case of the study carried out with rats that were treated with azoxymethane (AOM), a carcinogen for CRC, showed that germ-free rats had a lower rate of cancer development and a higher immune response for CRC compared to conventional rats [234].

In view of all that has been said and supported by reviews in which it is stated that the development of tumorigenesis may be due to effects of the microbiota such as inflammation, bacterial toxicity, alteration of the intestinal barrier and the immune system [235] or to the fact that obesity and dysbiosis are correlated [236] so the existence of a link between obesity, microbiota and CRC could be affirmed.

4. MODULATING MICROBIOTA FOR COLORECTAL CANCER PREVENTION

Modifiable dietary factors are largely responsible for the incidence of CRC in a population, to such an extent that in studies conducted with immigrants it was observed that people who moved to countries with a high rate of CRC quickly acquired that higher rate of CRC risk [32, 33].

Some of the components that are part of food are capable of influencing the intestinal microenvironment favoring different bacterial groups. In turn, these changes in the gut microbiota can affect the risk rate for CRC. That is why some compounds could be used to some extent as bacterial modulators for the prevention of CRC.

4.1. FIBER

The metabolic activities of the digestive tract have been shown to be positively influenced by dietary fiber [79, 237]. The hypothesis that dietary fiber affects the rate of CRC

development appears to originate from the study by Burkitt *et al*. in which the relationship between a high fiber diet and a low prevalence of CRC in many African countries [40, 77, 238] was shown. Being diverse the observational studies that agree with the protective effect of the fiber of the diet in the CRC [40]. The European Prospective Research on Cancer and Nutrition (EPIC) study concluded that the risk of developing CRC could be reduced by up to 40% by increasing fiber intake [239]. This same observational study, EPIC, determined that the inverse relationship between fiber intake and incidence of CRC was independent of the source of fiber ingested [240]. However, a meta-analysis of observational studies inversely associated the risk of CRC with fiber from cereals but not with fiber from fruits, vegetables, or legumes [82]. After monitoring more than 10,000 participants for 15 years, the National Institutes of Health and American Association of Retirees (NIH-AARP) Diet and Health Study confirmed the inverse relationship of dietary fiber intake from whole grains with CRC risk, a reduction of up to 16%, but not for other fiber sources [241].

It has been determined that in order for dietary fiber to influence the body, the food eaten must provide at least 25 g/day of fiber [242]. Dietary fiber favors an increase in stool volume, a decrease in transit time and, therefore, reduces the time of exposure of the colorectal epithelium to carcinogens that pass through the intestine [78]. Colon health is also supported by dietary fiber due to its relationship with the gut microbiota. The action of the intestinal microbiota on dietary fiber may be related to its anti-CRC effect [238]. The undigested soluble fiber is fermented in anaerobiosis and SCFAs (mainly butyrate, propionate and acetate) are produced [79, 243], with the known protective effect of SCFAs on the mucosa of the colon, especially butyrate [243]. The SCFAs produced regulate colonic metabolism and the immune system and reduce the risk of CRC. The fiber supplementation in the diet produces an effect on the intestinal microbiota, the

populations of *Bifidobacterium* and *Lactobacillus* spp. [238] lactate and acetate producers. Furthermore, some butyrate-producing bacteria, *Eubacterium rectole*, *Roseburia* spp, and *Faecalibacterium prausnitzii* increase in the colon [238]. During this fermentation the fecal pH is reduced, therefore the bacterial carcinogens derived from the metabolism of bile acids also decrease [243].

But increased dietary fiber intake is not only related to a lower risk of CRC, but also to an improvement in survival rate after diagnosis [84]. Whole grains are considered the most important source of dietary fiber inversely associated with the risk and mortality of CRC [82, 84, 243].

Finally, dietary fiber is also attributed a chemoprotective effect, where different sources of fiber may have a greater or lesser protective effect in different locations of the colon [243].

4.2. POLYUNSATURATED FATTY ACIDS

The components of the diet that have the greatest impact on the development of tumors are fat and fiber, of which the type and origin are very important for their greater or lesser influence on tumorigenesis [244]. Current preclinical and epidemiological studies support the theory that polyunsaturated fatty acids (PUFAs), especially omega 3 polyunsaturated fatty acids (PUFA ω-3), have preventive effects for CRC [245]. At the end of the 20th century, Scholss *et al*. observed the lowest incidence of CRC among fisher populations [106]. Shortly thereafter, Rose and Connolly encouraged the implementation of clinical trials with PUFA ω-3 supplementation, recognizing its effect on reducing the risk of development of some cancers, including CRC [246].

More than 520,000 subjects participated in the European EPIC study, in which a wide variation in fish intake was used and circulating levels of PUFA ω-3 and its relationship with the risk of CRC were observed, confirming an inverse relationship between the fish consumption and CRC [239]. The follow-up of this cohort for 11 more years associated the intake of 100-200 g/week of fish or the intake of dietary PUFA ω-3 with a 7% reduction in the risk of CRC, although circulating PUFA ω-3 did not were associated with CRC, these relationships were independent of the type of fish consumed, fatty or lean [247].

The importance of PUFAs on the health of the individual is based on the direct antitumor activity, the anti-inflammatory properties and the improvement of the antitumor immune response [248], being more important in the large intestine due to the greater influence of ingested substances [249]. The importance of PUFAs at this level of the digestive tract has also been seen, since it is where they have an impact on the intestinal microbiota [250]. In contrast to saturated fats that alter the intestinal microbiota in a negative way for health, PUFAs produce favorable effects [244]. The supplementation of the diet with PUFAs in healthy individuals produces a decrease in *Faecalibacterium* and an increase in *Bacteroidetes* [251] and bacteria producing SCFAs, mainly of butyrate. SCFAs promote apoptosis of colorectal epithelial cells and enhance the antitumor immune response [248]. When butyrate is metabolized, it gives rise to ROS, which affects dietary PUFAs that are incorporated into cell membranes and which are susceptible to being oxidized due to their high degree of unsaturation [244]. Apoptotic factors are released from the mitochondria to the cytosol as a consequence of lipid oxidation. All this added to the fact that the cancer cells of CRC preferentially uptake PUFAs [195] shows one of the possible mechanisms of action by which the anticancer effect of this type of fatty acids occurs.

The presence of eicosapentaenoic acid and docosahexaenoic acid in the diet produces an increase in *Lactobacillus* and *Bifidobacteria* and a reduction in *Helicobacter* and *Fusobacteria nucleatum*. *Lactobacillus* and *Bifidobacteria* are inversely associated with inflammation and *Helicobacter* and *Fusobacteria nucleatum* are opportunistic pathogens [244]. PUFAs favor the development of beneficial bacterial populations in the digestive tract and the reduction of pathogens, so that dysbiosis of the intestinal microbiota is reduced [252].

The regulation of differentiation and apoptosis of colonocytes are the main mechanisms through which PUFAs exert their anticancer role [253, 254]. A reduction of proliferating cells and an increase in apoptosis in tumor cells was observed in C57BL/6J mice with CRC induced by azoxymethane-dextran sodium sulfate after treatment with eicosapentaenoic acid, in addition to an increase in Lactobacillus [255].

In APC/Min+ mouse model of spontaneous intestinal cancer, polyposis was reduced when treated with a mixture of PUFAs [256, 257]. Dietary supplementation with eicosapentaenoic acid decreased the incidence of tumors in chemically induced CRC model mice [256] and suppressed liver metastasis in BALB/c mice [258]. It has also been observed that PUFA ω-3 can improve the action of anticancer treatments and reduce their toxicity. The tumor growth of the HCT116 xenograft was jointly inhibited by oxaliplatin and docosahexaenoic acid [259]. Currently, PUFA ω-3 supplementation has been proposed as an adjuvant in the treatment of CRC [260]. It has been seen *in vitro* that docosahexaenoic acid acts on the toxicity of arabinosylcytosine, causing it to act only in tumor cells [261]. Or like rats with colon cancer and subjected to treatment with 5-FU, they decreased the growth of tumors and increased chemosensitivity after being supplemented with fish oil [262]. The reduction in tumor burden and DNA damage and

the increase in apoptosis associated with 5-FU treatment has been enhanced by PUFAs [263].

Dietary PUFAs have also been associated with decreased microsatellite instability (reduced CRC risk) [247] and with improvement in the DNA repair system [264].

And supplementation with PUFA ω-3 pre- or post-operatively significantly improved the inflammatory and immune responses after CRC resection [260].

4.3. POLYPHENOLS

Dietary polyphenols are present in a wide variety of foods such as fruits, vegetables, cereals, nuts, wine and tea, and are widely related to chemical protection both in animal models and in human epidemiological studies [194]. The biological activities of many pathologies are enhanced by this family of natural compounds, such as diabetes, diseases related to obesity and inflammation, cardiovascular diseases, neurodegenerative diseases and cancer [194, 265]. Polyphenols have been much investigated lately for their protective effect on CRC [266]. The required amount of phenolic compound (10 mL/kg of dietary fiber) that could reduce the risk of CRC by modifying the expression of oncogenes, tumor suppressor genes, apoptosis and even affecting the cell cycle was estimated [267]. The modulation of the immune system [268] and antioxidant, anti-inflammatory and anticancer properties are attributed to polyphenols, in addition to the indirect effects derived from the modulation of the intestinal microbiota [269].

Insoluble polyphenols reach the colon, these individual polyphenols have beneficial effects on the gastrointestinal tract [270], but are also metabolized by intestinal bacteria and derived metabolites have positive health effects at the systemic level [269]. But polyphenols also have an antibacterial function against certain pathogens [265, 268] and

a prebiotic effect that favors the population of *Lactobacilli* and *Bifidobacteria* [271]. This turns the relationship between polyphenols and microbiota into a bidirectional interaction, where polyphenols affect the composition of the intestinal microbiota [271] and in turn, this microbiota metabolizes polyphenols which will have other effects. *Clostridium* spp. (*C. histolyticum*), *Pseudomonas* spp., *Salmonella* spp., *Bacillus* spp., *Escherichia coli*, *Helicobacter pylori* are among the bacteria that see their populations reduced as a consequence of polyphenols, while *Lactobacillus* spp., *Bifidobacterium* spp., *Akkermansia* spp. (*A. muciniphila*) and *Faecalobacterium* spp. (*F. prausnitzii*) increase within the intestinal microbial community, some of them being capable of metabolizing the polyphenols present [268].

The antioxidant action of polyphenols causes these compounds to affect different metabolic pathways, such as cytochrome P450 and signaling pathways mediated by MAPK, Phosphoinositide 3 - kinases (PI3K), ROS, NF-kB and Insulin-like growth factor 1 (IGF-1), either in normal or pathological processes of cells. Phenolic derivatives focus their action on cell adhesion, cell migration and tumor angiogenesis [224]. With regard to inflammation, polyphenols are also capable of acting on it by reducing the nuclear factor NF-kB, modulating the signaling pathways that associate inflammation and cancer [265]. In a study in which the phenolic compounds in olive oil were tested, it was seen that they were capable of affecting the inflammatory processes of the colon mediated by NF-kB, inducible nitric oxide synthase, IL-8 and IL-6 [272]. In dextran sodium sulfate (DSS) induced mice, the antioxidant response was improved and the levels of inflammatory markers, TNF-α, IL-6 and serum amyloid A, were reduced after being treated with tea polyphenols [265]. As well as the phenolic compounds of blueberries, grapes or cocoa demonstrated their antitumor activity, suppressing the expression of

various cytokines (IL-1β, TNF-α, IL-6, IL-17, Interferon gamma (IFN-γ)), in addition to reducing the infiltration of CD3 T cells in colon tissue [268].

In various studies with colon cells HCT116, it has been seen how the polyphenols of *Pleurotus eryngii* (edible fungus) reduce the proliferation of cells [265], epigenin was related to the blocking of the cell cycle, the induction of apoptosis and the inhibition of autophagy [273] or how curcumin induced DNA methylation in this cell line [270]. Although it has been seen that the polyphenols with the greatest potential to alter methylation by inhibiting the methyltransferase enzyme are the polyphenols from green tea [270]. The beneficial effect of phenolic compounds in cancer pathology is observed in the inhibitory action of the early or advanced stages and of the metastatic process [224].

The chemoresistance and toxicity of CRC therapies make treatment of the disease difficult [265]. Currently, beneficial effects have also been detected at this level, where some polyphenols reduce the methylation of micro-RNA 149 (miR-149). And miR-149 can stop the cell cycle, and decrease the expression of cyclin B1, Serine/threonine kinase (AKT) and Cyclin-dependent kinase 1 (CDK1), improving chemosensitivity in CRC treatments [274].

5. GUT MICROBIOTA ON THE RESPONSE TO CRC TREATMENT AND ITS TOXICITY

Currently, preoperative radiochemotherapy has become a standard process in the treatment of CRC [275, 276]. There are more and more studies in which the gut microbiota is related to the efficacy and/or toxicity of chemotherapeutic and immunotherapeutic treatments [277, 278]. Being translocation, immunomodulation, metabolism, ecological variation and reduction of diversity the mechanisms through

which the intestinal microbiota influences the response to drugs and toxicity in patients with CRC [279] (**Figure 5**.).
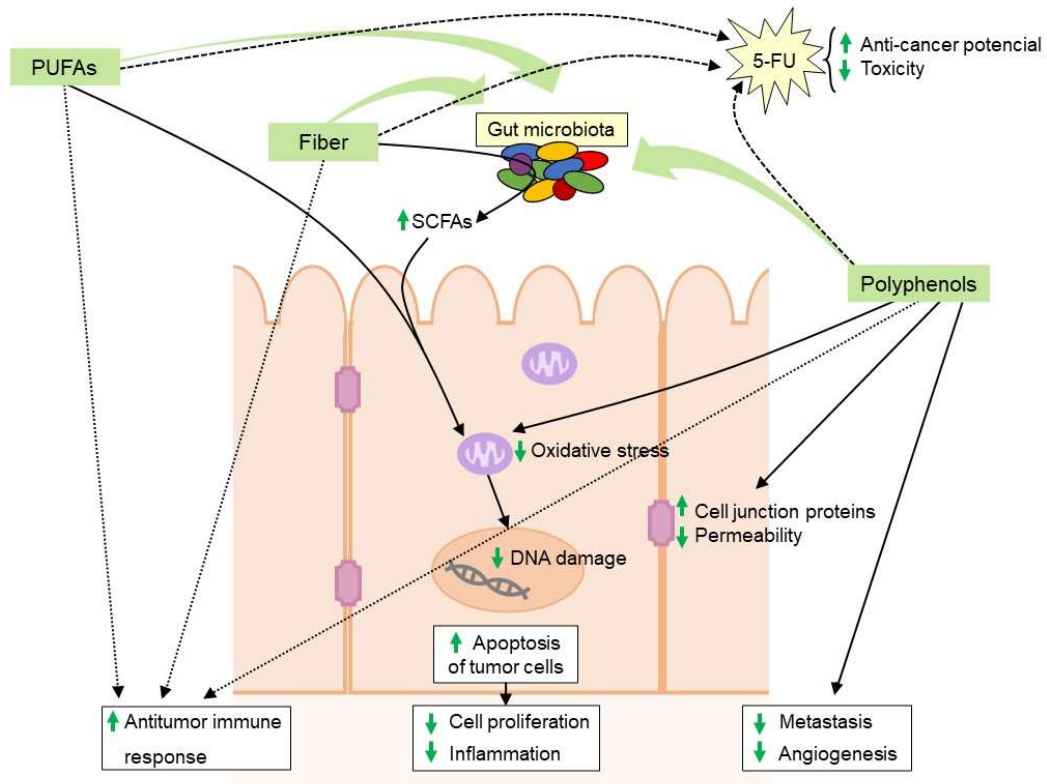
Figure 5. Beneficial effects of dietary supplementation with PUFAs, polyphenols and fiber on the intestinal microbiota and colon cells for the reduction of CRC risk or to enhance the response to cancer therapy when are used as adjuvant to conventional treatment. PUFAs: polyunsaturated fatty acids; 5-FU: 5-fluorouracil; SCFAs: short chain fatty acids.

Dysbiosis of the gut microbiota has been suggested as an influence on the response of the host to various cancer therapies, whether chemotherapy, radiotherapy or immunotherapy [280, 281]. Intestinal microbiota modulated the efficacy and toxicity through key mechanisms, such as the translocation, immunomodulation, metabolism, reduced diversity, and ecological variation [282].

As seen in a study by Yuan *et al*. in which the efficacy of 5-FU treatment was diminished due to dysbiosis caused by the use of antibiotics [283]. In addition to affecting the efficacy of chemotherapy, antibiotic-induced dysbiosis prior to 5-FU treatment was also associated with worse overall survival in patients with metastatic CRC [284].

Several bacterial phyla are known for their influence on drugs, either by physical adherence to them or by participating in their metabolism, through proteolytic degradation reactions, isoxazole cleavage, denitrification, deconjugation, acetylation/deacetylation, amine formation and/or hydrolysis [285].

In mice with CRC, intestinal mucosis induced by 5-FU/oxyliplatin was reduced thanks to *L. casei* variety *rhamnosus* [286]. The frequency of diarrhea and abdominal distress and avoided the dose reduction caused by intestinal toxicity was decreased in patients with *L. rhamnosus* supplementation compared to patients taking placebo [287]. For this reason, more and more studies use probiotics in relation to radiotherapy treatments, since certain intestinal bacteria participate in the repair of injuries and reduce the incidence and severity of diarrhea derived from the treatment [288]. Approximately forty chemotherapeutic drugs have been shown to be metabolized by the gut microbiota [289].

HYPOTHESIS

Previous studies have demonstrated that patients with CRC present a different intestinal microbiota and capable of carrying out different functions than healthy subjects, and these differences may be one of the causes responsible for the development of cancer. In addition, the presence of obesity is an important risk factor associated with the development of CRC. Moreover, the dysbiosis associated with obesity can cause physiological changes that could increase the risk of cancer. In fact, obesity is a low-grade inflammatory state and tumorogenesis is favored by inflammation, consequently, obesity can increase the risk of CRC. The presence of obesity in patients with CRC is associated with changes in the composition of the intestinal microbiota, which entails a different systemic inflammatory response compared to CRC patients without obesity and healthy controls, this situation may influence the immune response against the tumor and in its development. Then, obese patients with CRC have a different gut microbiota capable of carrying out different functions from healthy lean subjects and lean CRC patients. Finally, there is a bidirectional interaction between the neoadjuvant radiochemotherapy (RCT) and the gut microbiome in CRC patients: RCT might induce alterations in the gut microbiome; and that these alterations might in turn influence the effectiveness of RCT by directly interact with the treatment and/or by stimulating the host's immune response. The intestinal microbiota also produces important molecules such as SCFAs, TMAO and polyamines, which can be beneficial or harmful for the oncological process, which would demonstrate that the microbiota could play an important role in the evolution of CRC patients subjected to neoadjuvant RCT. Further, in patients with CRC, intestinal permeability increases after treatment with RCT, possible due to the loss of a specific commensal microbiota responsible for increasing the expression of tight junction proteins and contributing to maintaining the integrity of the intestine.

OBJETIVES

Study 1

1. To determine the gut microbiota composition and functions in fecal samples from CRC patients with (OB-CRC) and without obesity (L-CRC).

2. To compare the gut microbiota profile present in OB-CRC and L-CRC with the microbiota present in non-obese healthy controls (L-HC).

3. To analyze the fecal levels of the microbial-derived metabolite TMAO in the study groups.

4. To study the intestinal permeability and inflammatory status in the study groups.

5. To unravel the possible relationship between gut microbiota and microbial-derived metabolite TMAO, the inflammatory status, and the intestinal permeability in the context of obesity-associated CRC.

Study 2.

1. To stablish the differences in gut microbiota composition and diversity between CRC patients and healthy controls.

2. To study the changes in diversity and composition of the intestinal microbiota of CRC patients before, during, and after completing the neoadyuvant radiochemotherapy treatment.

3. To analyzed the differences in gut microbiota composition and function after the classification of CRC patients in good (responders) and poor or non-responders to treatment (non-responders).

4. To measure metabolites produced by the intestinal microbiota such as short-chain fatty acids and polyamines before and after radiochemotherapy in responders and non-responders and study their relationship with the composition of their intestinal microbiota

5. To measure markers of inflammation and intestinal permeability in plasma samples from responders and non-responders at baseline and at the end of radiochemotherapy treatment and study their relationship the composition of their intestinal microbiota.

6. To establish if initial microbiota composition could predict response to radichemotherapy treatment in CRC patients.

MATERIALS AND METHODS

1. STUDY PATIENTS

- **Study 1**

Forty-five patients aged 35-75 years with stages II–III (T2-T4 and/or N1-N2) were recruited at the Radiotherapy Oncology Service at Virgen de la Victoria Hospital. Patients were enrolled at initial diagnosis and did not receive any treatment before collection of fecal and peripheral blood samples. Patients were dichotomized into non-obese (BMI < 30 kg/m^2) (L-CRC) and obese (BMI $\geq$ 30 kg/m^2) (OB-CRC) according to the WHO guidelines. Exclusion criteria were familial CRC, presence of inflammatory bowel disease, food allergies, use of antibiotics within the past 3 months before sampling, or regular use of non-steroidal anti-inflammatory drugs, statins, or probiotics.

Additionally, 20 non-obese healthy controls (L-HC) (BMI < 30 kg/m^2) (age- and gender-matched controls) were recruited for the study. The exclusion criteria for healthy controls included gut disease diagnosis and/or medication, and previous CRC diagnosis.

- **Study 2**

Forty patients aged 35-75 years, who were newly diagnosed with CRC in stages II-III (T2-T4 and/or N1-N2) from the Radiotherapy Oncology Service at the Virgen de la Victoria Hospital were enrolled in the study and were followed-up for at least 1 year. All the CRC patients received only neoadjuvant treatment for 5 weeks with pelvic radiation therapy (50 Gy in fractions of 2 Gy/session) and oral Capecitabine (825 mg/m^2/12h) during radiotherapy treatment. Patients with a history of CRC or bowel resection, chronic inflammatory bowel disease and hereditary colorectal cancer syndromes were excluded from the study. Patients who received pelvic cancer radiation therapy in the previous 2 years, used antibiotics in the previous 2 months, or regularly used non-steroidal anti-

inflammatory drugs, statins or probiotics before the study were also excluded. A pathologist examined surgical specimens and tumor response after neoadjuvant radiochemotherapy was determined in surgical specimens according to the tumor regression grades (TRG) system described by Mandard *et al.* [290]. We divided CRC patients into TRG1-2 (patients with good response or responders (R)) and TRG 3-5 (patients with poor or non-response (NR)). Blood and feces samples were collected at baseline (T0), 2 and 4 weeks after starting radiochemotherapy (T1 and T2, respectively), and 7 weeks after finishing treatment (T3).

In the study we also included fecal samples from 20 healthy patients matched ac-cording to sex, age and BMI with CRC patients. Healthy controls did not have gastro-intestinal tract disorders or other complications and were not administered antibiotics or probiotics during the 2 months prior to sample collection.

The study protocol was approved by the Medical Ethics Committee at the Virgen de la Victoria University Hospital and conducted in accordance with the Declaration of Helsinki. Written informed consent was provided by all study participants.

2. LABORATORY MEASUREMENTS

Fasting venous blood samples were collected and centrifuged to 4000 × g for 15 min isolate the plasma and serum that will be aliquoted and stored at -80ºC until analysis. Levels of serum glucose, total cholesterol, triglycerides, high density lipoprotein (HDL) cholesterol, low density lipoprotein (LDL) cholesterol and a glycated hemoglobin (HbA1c) were measured in duplicated using a Dimension autoanalyzer (Dade Behring Inc., Deerfield, IL) by enzymatic methods (Randox Laboratories Ltd., UK).

3. DNA EXTRACTION AND GUT MICROBIOTA SEQUENCING

The walnut-sized stool samples will be collected in the morning before breakfast in a sterile wide-mouth flask and frozen as soon as possible at -80ºC until further analysis. Thawing of the samples will take place gradually over 24 hours at 4ºC to minimize the possible loss of the bacterial groups most sensitive to temperature changes.

DNA extraction from 200 mg of faecal material was performed using the QIAamp DNA stool Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. DNA concentration was determined by absorbance at 260 nm (A260), and purity was estimated by determining the A260/A280 ratio with a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

DNA was amplified using the Ion 16S Metagenomics kit (Thermo Fisher Scientific, Madrid, ES) that contains a primer pool to amplify multiple variable regions (V2, 3, 4, 6-7, 8 and 9) of the 16S rRNA gene. The Ion Plus^{TM} Fragment Library Kit (Thermo Fisher Scientific, Madrid, ES) was used to ligate the barcoded adapters to the generated amplicons and create the barcoded libraries, which were pooled and templated on the automated Ion Chef system (Thermo Fisher Scientific, Madrid, ES). The sequencing was done on an Ion S5 platform (Thermo Fisher Scientific, Madrid, ES).

4. INTESTINAL PERMEABILITY ANALYSIS

Plasma level of zonulin was determined by enzyme linked immunosorbent assay (ELISA) using commercial kits (Immunodiagnostik AG, Bensheim, Germany). Measurements were done in duplicate, and mean values were used for analysis. The detection limit for zonulin was 0.22 ng/mL. Intra and interassay coefficients of variation were between 3-10%.

5. CYTOKINE ANALYSIS

Serum levels of IL-10 and IL-1β were measured by ELISA assays (Novex, Life Technology). Detection ranges were 7.8-500 and 3.9-250 pg/mL for IL-10 and IL-1β, respectively.

6. QUANTIFICATION TRIMETHYLAMINE N-OXIDE IN SERUM SAMPLES

Trimethylamine N-oxide was quantified in serum samples using Nuclear Magnetic Resonance (NMR) (Embade *et al.*, 2016). For NMR analysis, serum samples were thawed for 30 min and aliquots of 300 μL were mixed with 300 μL of phosphate buffer (pH 7.0) containing 5 mmol/L Trimethylsilyl propionate and 5% v/v Deuterium oxide (D2O). The final mixtures were gently shaken and transferred to NMR 96 rack tubes. NMR spectra were measured at 300 K on a Bruker Avance IVDr 600 MHz spectrometer (Bruker Biospin, Germany) and a Sample Jet Robot (Bruker Biospin, Germany) was used for automatization of the measurements. For each sample, three complementary NMR spectra were recorded. A standard 1H spectrum with water suppression (Nuclear overhauser effect spectroscopy (NOESY)) was assessed. We repeated the same experiment with an appended T2 relaxation filter implemented as a CPMG module to decrease broad signals from proteins and lipoproteins. Finally, a two dimensional 1H,1H JRES was measured to help with the identification of the metabolites. All spectra were acquired and processed within the TopSpin program (TSP) (Bruker Biospin, Germany) applying an automatic phase correction and referenced against internal TSP (δ = 0.00 ppm). To identify and quantify the desired metabolites (TMAO) different amounts of these compounds were added to the serum samples, were measured by NMR and the values of the intensity peaks were represented against concentration. The pure metabolite molecules used for referencing were all obtained from Sigma-Aldrich (St. Louis, MO,

United States). For all the spectra we measured the intensity of the peaks corresponding to TMAO (singlets) and the concentrations in the samples were calculated with the power fitted calibration curves.

7. ANALYSIS OF SERUM POLYAMINE LEVELS BY ULTRA-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

Fifty microliters of serum aliquoted in 1.5 mL Eppendorf LoBind tube were mixed with 5 µL of internal standard and 167 µL of methanol and vortexing for 1 minute for protein precipitation. Then, 334 µL of chloroform was added to the mixture, vortexed for 1 min and centrifuged for 10 min at 15,000 rpm and 4°C. The upper layer was collected and transferred to a new tube. In order to derivatize the sample, 100 µL of carbonate-bicarbonate buffer (pH 9) and 50 µL of dansyl chloride (10 mg/mL in acetone) were added. The mixture was vortexed and placed in the dark for 1 hour at room temperature. The compounds were extracted with 250 µL of ethyl acetate twice; prior to the second extraction, 2.5 µL of trifluoroacetic acid were added. The combined organic phases were evaporated in a SpeedVac at 45°C and stored at −20°C until analysis. Samples were reconstituted in 50 µL of ammonium acetate 0.2 M:acetonitrile (30:70).

Chromatography was performed with an Agilent ultra-high performance liquid chromatography (UHPLC) 1290 series binary pump (Agilent Technologies, Santa Clara, CA, USA), and separation was carried out on a Kinetex EVO C18 column (2.6 µm particle size, 2.1 mm internal diameter × 150 mm length) (Phenomenex, Torrance, CA, USA) held at 25°C. The mobile phase for elution was a gradient established between water acidified with 0.1% formic acid (A) and acetonitrile acidified with 0.1% formic acid (B) at a flow rate of 400 µL/min. The injected amount was 2.5 µL.

Tandem mass spectrometry (MS/MS) analysis was performed on an Agilent QqQ 6490 Series mass spectrometer operating in AJS + ESI. The ionization source parameters were optimized using MassHunter Optimizer (Agilent Technologies, version 6.0) as follows: nebulizer gas (nitrogen) with a pressure of 15 psi, a gas flow of 15 L/min at 200°C, a sheath gas flow of 11 L/min at 350°C, a capillary voltage of 2.5 kV, and a nozzle voltage of 1000 V.

Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis was performed using an Agilent UHPLC 1290 Infinity II Series coupled to an Agilent QqQ/MS 6490 Series (Agilent Technologies, Sta. Clara, CA, USA). Chromatographic separation was performed using a Kinetex EVO C18 analytical column (2.6 µm; 2.1 mm × 150 mm) (Phenomenex, Torrance, CA, USA).

Quantification was performed with the commercial standards arginine, ornithine, N1-acetylspermidine (N1-AcSPD), N8-acetylspermidine (N8-AcSPD), N-acetylputrescine (N1-AcPUT), N-acetylspermine (N1-AcSP), spermine (SP), spermidine (SPD), putrescine (PUT), N1, N12-diacetylspermine (N1,N12-DiAcSP), and N1, N8-diacetylspermidine (N1,N8-DiAcSPD) (Toronto Research Chemicals, North York, ON, Canada). Amino acid internal standards were lysine (13C6, 15N2) and arginine (13C6, 15N4) (Cambridge Isotope Laboratories) and for polyamines spermine-d20, spermidine-d6, putrescine-d8 and N8-acetylspermidine-d3 (Toronto Research Chemicals).

8. EXTRACTION AND ANALYSIS OF SHORT CHAIN FATTY ACIDS FROM FECAL SAMPLES BY GC-FID

After thawing, fecal samples were homogenized with the help of a spatula and aliquot of 100 mg were acidified with 0.25 mL of sulfuric acid 50% w/v. The solution was vortex for 3 min and 50 µL of internal standard solution were added. The organic phase

extraction was done by adding 1 mL of ethyl ether and centrifuging for 5 min at 2800 × g. The organic phase was collected into another vial. At the end, 0.5 µL of the collected organic phase were injected into the GC for analysis. The analysis was done using a gas chromatograph Agilent Technologies 6850 GC (Santa Clara, CA, USA) equipped with a split/splitless injector and FID (Agilent Technologies, Santa Clara, CA, USA). The identity of SCFAs detected in the fecal samples was confirmed by comparison of their retention times and their mass spectra with those of the analytical standards (Sigma–Aldrich).

9. BIOINFORMATICS ANALYSIS

Analysis of 16S rRNA amplicons was done with QIIME 2-2019.4. Raw sequence data were quality filtered and denoised, dereplicated and chimera filtered using the q-dada2 plugin with DADA2 pipeline. Q2-feature-table plugin was used to merge into a single feature table the sequence variants obtained by DADA2 pipeline. All amplicon sequence variants from the merged feature table were clustered into OTU's (Operational taxonomic units) with Open Reference Clustering method against the Greengenes version 13_8 with 97% of similarity from OTUs reference sequences using the q2-vsearch plugin with 97% similarity of sequence. The OTUs were aligned with MAFFT (via q2-alignment) and used to construct a phylogeny with fasttree2 (via q2-phylogeny). Taxonomy was assigned to OTUs using the q2-feature-classifier classify-sklearn naïve Bayes taxonomy classifier. Alpha diversity metrics (Shannon and Chao1), beta diversity metrics (Bray Curtis dissimilarity), and Principal Coordinate Analysis (PCoA) were estimated using q2-diversity after samples were rarefied to 994 sequences per sample. Alpha diversity significance was estimated with Kruskal Wallis test using q2-diversity plugin. Beta diversity significance was estimated using ANOSIM.

10. STATISTICAL ANALYSIS

The Kruskal-Wallis rank-sum test was used to compare the bacterial abundance between study groups and false discovery rate (FDR) using the Benjamini-Hochberg method was applied to correct the significant p-values ($q < 0.05$). Kruskal-Wallis rank-sum test and subsequent post hoc Bonferroni, analyzed differences in the clinical and biochemical variables between three study groups and the differences between two groups were analyzed using Mann-Whitney U test. Inter-group comparison among post-treatment changes in polyamines, SCFAs and zonulin by a covariance model (ANCOVA) adjusted for baseline were carried out. Wilcoxon signed-rank test was used to calculate differences in SCFAs, polyamines and zonulin between baseline and post-treatment timepoint T3 (study 2). The Spearman correlation coefficients were calculated to estimate the correlations between bacterial taxa and gut microbiota derived-metabolites and permeability. A linear regression analysis was done to identify what bacteria was an independent predictor for serum inflammatory mediators, TMAO, and zonulin levels in each study group (study 1). Statistical analyses were carried out with the statistical software package SPSS version 26.0 (SPSS Inc., Chicago, IL, United States). Random forests (RF) were used to predict baseline bacteria (species-level relative abundance data) related to the neoadjuvant RCT response using the default parameters of the R implementation of the algorithm (R package "randomForest"), and bootstrapping (n = 500) was used to assess the classification accuracy. Values were considered to be statistically significant when the $p < 0.05$.

RESULTS

1. STUDY 1: Gut microbiota-mediated inflammation and gut permeability in patients with obesity and colorectal cancer

1.1. CLINICAL CHARACTERISTICS OF THE PATIENTS AND HEALTHY CONTROLS

Table 1. Clinical and biochemical characteristics, serum levels of trimethylamine N-oxide (TMAO) and inflammatory markers in the study groups.

	L-HC N = 20	L-CRC N = 23	OB-CRC N = 22	p
Age (years)	61.42 ± 7.40	62.52 ± 7.99	64.43 ± 7.31	0.208
Gender, n (M/F)	10/10	12/11	11/11	
BMI (kg/m^2)	25.45 ± 3.23[a]	25.32 ± 3.67[a]	35.82 ± 3.83[b]	0.001
Constipation, n (%)	6 (20%)	8 (26.6%)	10 (33.3%)	0.383
Alcohol consumption, n (%)	4 (13.3%)	4 (13.3%)	3 (10%)	0.997
Current smoking, n (%)	9 (30%)	12 (40%)	10 (33.3%)	0.588
Biochemical data				
Glucose (mg/dL)	94.85 ± 9.86	92.04 ± 10.91	108.42 ± 10.53	0.456
Total cholesterol (mg/dL)	175.2 ± 33.6	187.12 ± 20.74	193.09 ± 19.91	0.325
Triglycerides (mg/dL)	112.67 ± 34.51	110.32 ± 33.03	127.7 ± 22.6	0.510
HDL-cholesterol (mg/dL)	60.7 ± 15.1	54.84 ± 18.41	47.28 ± 15.6	0.755
LDL-cholesterol (mg/dL)	107.78 ± 27.12	109.84 ± 25.98	112.80 ± 25.34	0.678
IL-1B (pg/mL)	76.40 ± 9.81[a]	103.32 ± 9.43[b]	110.65 ± 12.98[c]	0.006
IL-10 (pg/mL)	155.19 ± 9.60[a]	121.96 ± 15.22[b]	102.21 ± 9.82[c]	0.004
TMAO (ng/mL)	12.72 ± 9.57[a]	20.07 ± 15.23[b]	26.57 ± 14.95[c]	0.003
Histological variables				
Stages				
II		10 (43.47%)	12 (54.54%)	0.998
III		13 (56.52%)	10 (45.45%)	0.997
Tumor depthpenetration (T)				
T2-T3		14 (60.86%)	15 (68.18%)	0.775
T4		9 (39.13%)	7 (31.81%)	0.768
Grade of differentiation				
G1		9 (39.13%)	10 (45.45%)	0.995
G2		6 (26.08%)	5 (22.72%)	0.438
G3		4 (17.39%)	5 (22.72%)	0.998
No differentiation		3 (13.04%)	2 (9.09%)	0.997

BMI: body mass index; HDL: high density lipoprotein; LDL: low density lipoprotein TMAO: trimethylamine N-oxide; IL-1β: interleukin-1 beta; IL-10: interleukin 10. Values are expressed as mean ± SD. Different superscript letters indicate significant differences between study groups p < 0.05.

The clinical and anthropometric characteristics of study subjects are summarized in **Table 1**. No significant differences in age, gender, race, alcohol, and tobacco consumption, constipation, total cholesterol, triglycerides, HDL-cholesterol, LDL-cholesterol, fasting glucose, and HbA1c levels were found between study groups ($p > 0.05$).

As expected, significant differences were found in BMI between the CRC patients with obesity (OB-CRC) and non-obese healthy controls (L-HC). In addition, significantly higher levels of serum proinflammatory IL-1β and TMAO, and lower levels of anti-inflammatory IL-10 were found in the OB-CRC and non-obese CRC (L-CRC) groups compared to L-HC individuals. Moreover, these differences were even more pronounced when the OB-CRC patients were compared to L-HC subjects. No significant differences with respect to tumor stage and grade of tumor differentiation ($p > 0.05$) were found between L-CRC and OB-CRC groups.

1.2. RICHNESS AND DIVERSITY OF FECAL MICROBIOTA

A total of 5,206,881 good quality 16S rRNA gene sequences (average of 80,105.86 ± 34,581.61 sequences per sample) were obtained after trimming. The microbiota of all fecal samples after QIIME2 was composed of 2606 OTUS with a relative abundance higher than 1% in at least two samples (97% similarity cut-off). The Chao1 index (community richness) and Shannon index (microbiota diversity) were calculated at genus level to estimate the alpha diversity of the components of the fecal microbiota in the study groups. The Chao1 index values for each group suggested a significant decrease in richness in both CRC groups compared to healthy controls (L-CRC vs. L-HC, $p < 0.001$; OB-CRC vs. L-HC, $p = 0.035$) (**Figure 6A**). Moreover, a significant decrease in Shannon diversity was found in L-CRC and OB-CRC patients compared to L-HC controls (L-CRC vs. L-HC, $p = 0.0014$; OB-CRC vs. L-HC, $p = 0.039$) (**Figure 6B**). The alpha diversity

comparison revealed no different levels of diversity and richness between L-CRC and OB-CRC patients (Shannon: L-CRC vs. OB-CRC, $p = 0.34$; Chao1: L-CRC vs. OB-CRC, $p = 0.37$) (**Figure 6C**).
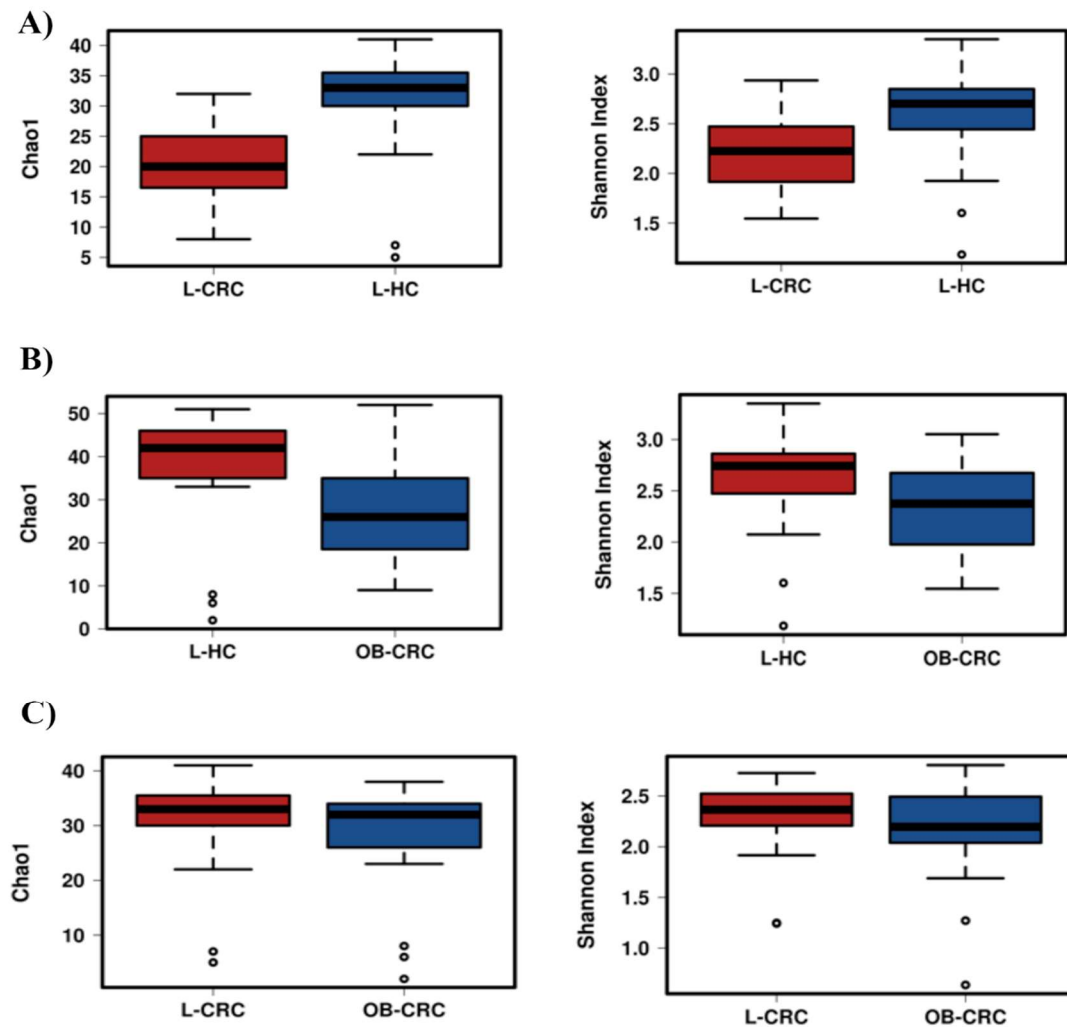
Figure 6. Richness (Chao1 index) and diversity (Shannon index) indices between microbial communities from feces of CRC patients with obesity (OB-CRC), non-obese CRC patients (L-CRC), and non-obese healthy controls (L-HC) at the genus level. (A) L-CRC vs. L-HC, (B) L-HC vs. OB-CRC, and (C) L-CRC vs. OB-CRC.

On the other hand, differences in microbiota communities (beta diversity) between study groups were determined by calculating the Bray-Curtis dissimilarity index. The ordination plots showed a significant separation in the bacterial communities in both L-

CRC and OB-CRC patients with respect to L-HC controls (L-CRC vs. L-HC, $p = 0.004$; OB-CRC vs. L-HC, $p = 0.007$, ANOSIM) (**Figure 7A, B**). Again, no significant differences in beta diversity was found between L-CRC and OB-CRC groups ($p = 0.485$, ANOSIM) (**Figure 7C**).
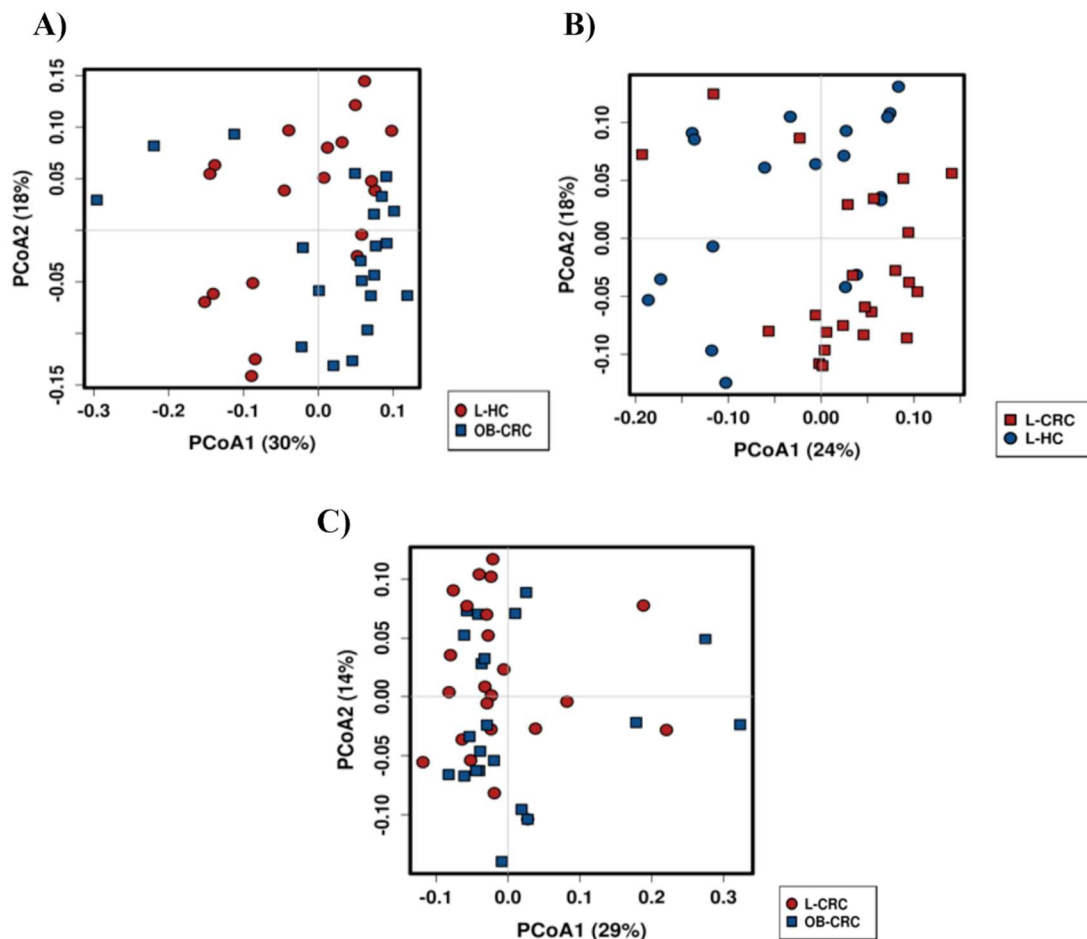
Figure 7. Clustering of fecal bacterial communities according to the different study groups by PCoA using Bray–Curtis dissimilarity matrix. Each point corresponds to a community coded according to the study groups. The percentage of variation explained by the plotted principal coordinates is indicated on the axes. (A) L-HC vs. OB-CRC, (B) L-CRC vs. L-HC, and (C) L-CRC vs. OB-CRC.

1.3. TAXONOMY OF FECAL MICROBIOTA IN THE STUDY GROUPS

The analysis of the distribution of the fecal microbiota at the phylum level indicated that Bacteroides and Firmicutes were the predominant phyla in the three study groups. Other

phyla such as Proteobacteria, Actinobacteria, Fusobacteria, and Lentisphaerae were also relatively abundant in all groups, while Tenericutes, Synergistetes Verrumicrobia, Spirochaetes, and WS6 were detected a low relative abundance (< 0.001; OB-CRC vs. L-HC, q = 0.005), Fusobacteria (L-CRC vs. L-HC, q = 0.002; OB-LCR vs. L-HC, q = 0.001), and Proteobacteria (L-CRC vs. L-HC, q = 0.012; OB-CRC vs. L-HC, q = 0.014), and a significant decrease in the abundance of Bacteroidetes (L-CRC vs. L-HC, q < 0.001; OB-CRC vs. L-HC, q = 0.005) in the CRC groups (non-obese and obese) compared to
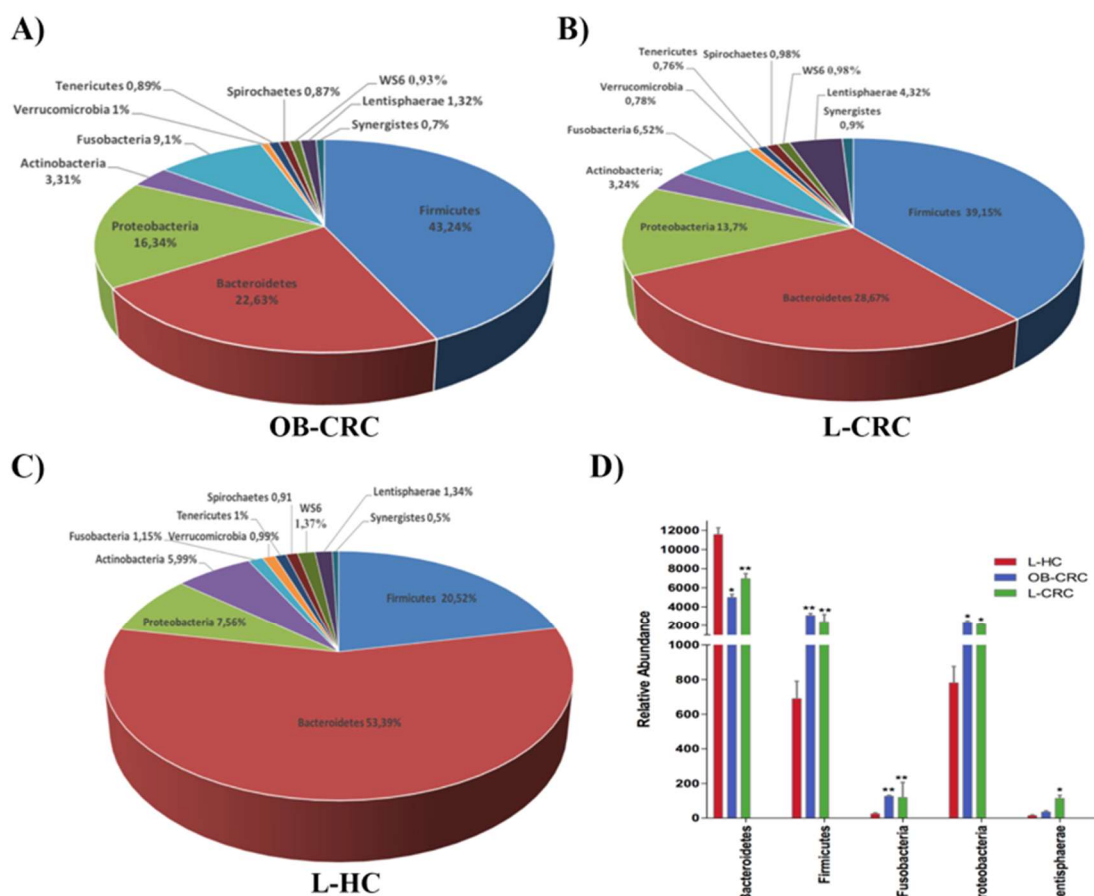
Figure 8. Phylum-level distributions of bacteria in fecal samples of (A) OB-CRC, (B) L-CRC, and (C) L-HC groups. Data are shown as a percentage of the total identified sequences per group. (D) Differentially abundant phyla in the stool samples of OB-CRC and L-CRC patients compared to L-HC * p < 0.05, ** p < 0.001. The bars indicate mean ± SD.

the non-obese healthy controls. Additionally, we found a significantly higher abundance of the phylum Lentisphaerae in L-CRC subjects compared to L-HC controls (q = 0.010). Significantly higher levels of Firmicutes (q = 0.008) and Proteobacteria (q = 0.013) were also found in the OB-CRC group with respect to the L-CRC group (**Figure 8**).

Twenty-seven bacterial families were detected in all study patients. Both CRC groups (L-CRC and OB-CRC) displayed a significantly higher abundance of the Fusobacteriaceae (L-CRC vs. L-HC, q = 0.002; OB-CRC vs. L-HC, q = 0.001), Prevotellacea (L-CRC vs. L-HC, q = 0.010; OB-CRC vs. L-HC, q = 0.003), Clostridiaceae (L-CRC vs. L-HC, q = 0.019; OB-CRC vs. L-HC, q = 0.020), Barnesiellaceae (L-CRC vs. L-HC, q = 0.002; OB-CRC vs. L-HC, q = 0.025), Porphyromonadaceae (L-CRC vs. L-HC, q = 0.010; OB-CRC vs. L-HC, q = 0.035), and Desulfovibrionaceae (L-CRC vs. L-HC, q = 0.005; OB-CRC vs. L-HC, q = 0.003) when compared to L-HC controls. Furthermore, a significantly lower abundance of Ruminoccocacea (L-CRC vs. L-HC, q = 0.018; OB-CRC vs. L-HC, q = 0.001) and Bacteroidaceae (L-CRC vs. L-HC, q = 0.002; OB-CRC vs. L-HC, q = 0.010) were found in the CRC groups with respect to the L-HC group. Victivallaceae was also significantly enriched in L-CRC patients compared to L-HC subjects (q = 0.010), while Enterobacteraceae (OB-CRC vs. L-CRC, q = 0.040; OB-CRC vs. L-HC, q = 0.029) and Streptococcaceae (OB-CRC vs. L-CRC, q = 0.004; OB-CRC vs. L-HC, q = 0.016) were significantly increased in the OB-CRC group compared to L-CRC and L-HC groups (**Figure 9**).

A)
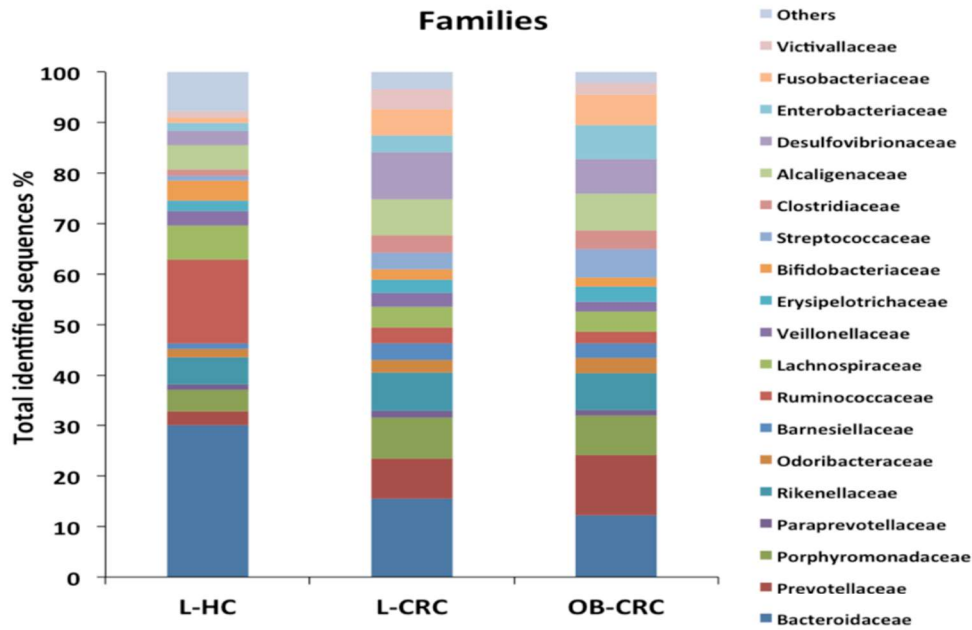
B)
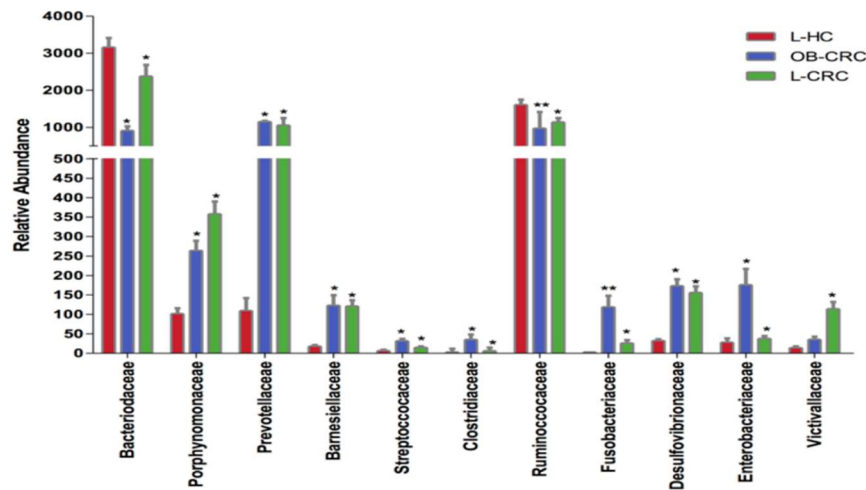
Figure 9. Family-level microbial classification of bacteria from OB-CRC, L-CRC, and L-HC stool samples. (A) Data are shown as a percentage of the total identified sequences per group. (B) Differentially abundant families in the stool samples of OB-CRC and L-CRC patients compared to L-HC * $p < 0.05$, ** $p < 0.001$. The bars indicate mean ± SD.

Further analysis revealed significant differences in the microbial composition at the genus level between the study groups. A total of 39 genera were identified among the 60 fecal samples, with only significant differences in 14 genera between CRC patients and healthy individuals. Thus, the genera *Prevotella* (L-CRC vs. L-HC, q = 0.001; OB-CRC vs. L-

HC, q = 0.003), *Clostridium* (L-CRC vs. L-HC, q = 0.019; OB-CRC vs. L-HC, q = 0.030), *Desulfovibrio* (L-CRC vs. L-HC, q = 0.002; OB-CRC vs. L-HC, q = 0.006) and *Enterococcus* (L-CRC vs. L-HC, q = 0.031; OB-CRC vs. L-HC, q = 0.05) were significantly increased in both CRC groups compared to the L-HC group. In addition, the relative abundance of *Bacteroides* (L-CRC vs. L-HC, q = 0.003; OB-CRC vs. L-HC, q = 0.045), *Butyricimonas* (L-CRC vs. L-HC, q = 0.001; OB-CRC vs. L-HC, q = 0.012), *Roseburia* (L-CRC vs. L-HC, q = 0.021; OB-CRC vs. L-HC, q = 0.019), *Ruminococcus* (L-CRC vs. L-HC, q = 0.018; OB-CRC vs. L-HC, q = 0.035), and *Alistipes* (L-CRC vs. L-HC, q = 0.005; OB-CRC vs. L-HC, q = 0.037) were significantly decreased in both CRC groups with respect to the L-HC group. Finally, other genera such as *Victivallis* was significantly elevated in the L-CRC compared to L-HC controls (q = 0.012). In OB-CRC patients we found that *Enterobacter* (OB-CRC vs. L-CRC, q = 0.038; OB-CRC vs. L-HC, q = 0.002), *Escherichia* (OB-CRC vs. L-CRC, q = 0.024; OB-CRC vs. L-HC, q = 0.006), *Fusobacterium* (OB-LCR vs. L-CRC, q = 0.003; OB-CRC vs. L-HC, q = 0.002), and *Streptococcus* (OB-CRC vs. L-CRC, q = 0.038; OB-CRC vs. L-HC, q = 0.05) were significantly enriched, while the relative abundance of *Blautia* (OB-CRC vs. L-CRC, q = 0.012; OBCRC vs. L-HC, q = 0.019) and *Faecalibacterium* (OB-CRC vs. L-CRC, q = 0.030, OB-CRC vs. L-HC, q = 0.024) were significantly lower when compared to L-CRC and L-HC individuals (**Figure 10**).

At the species levels, we found a significant rise in the abundance of *Enterococcus faecalis* (L-CRC vs. L-HC, q = 0.004; OB-CRC vs. L-HC, q = 0.012), and a significant decline in the abundance of *Bacteroides caccae* (L-CRC vs. L-HC, q = 0.007; OB-CRC vs. L-HC, q = 0.029), *Ruminoccocus lactaris* (L-CRC vs. L-HC, q = 0.05; OB-CRC vs. L-HC, q = 0.019), *Alistipes putredinis* (L-CRC vs. L-HC, q = 0.011; OB-CRC vs. L-HC, q = 0.05), and *Alistipes indistinctus* (L-CRC vs. L-HC, q = 0.008; OB-CRC vs. L-HC,
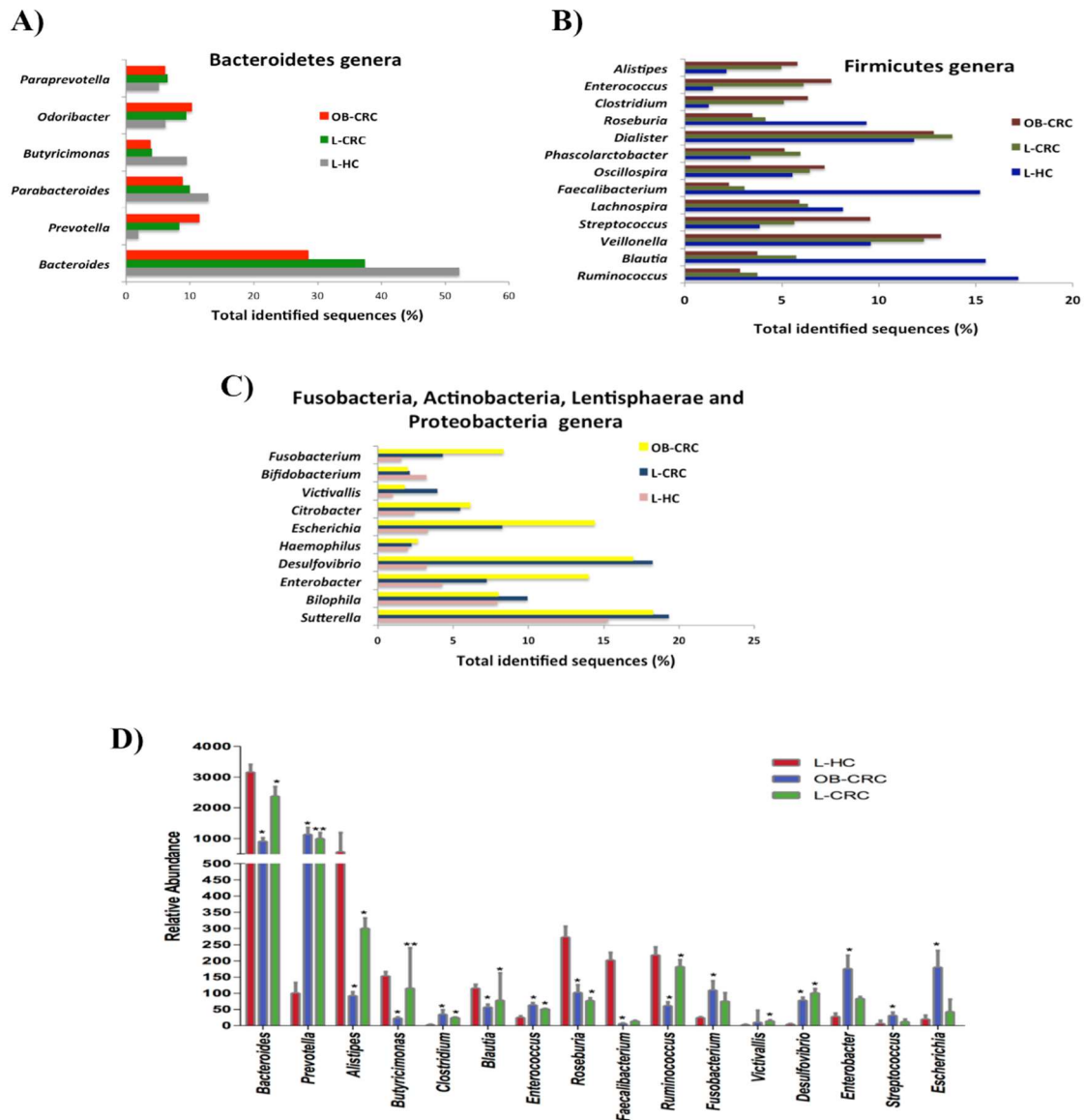
Figure 10. Relative abundance of bacterial genera in the microbiota of OB-CRC, L-CRC, and L-HC controls. (A) Bacteroidetes genera, (B) Firmicutes genera, (C) Fusabacteria, Actinobacteria, Lentisphaerae, and Proteobacteria genera. Data are shown as a percentage of the total identified sequences per group. (D) Differentially abundant genera in the stool samples of OB-CRC and L-CRC patients compared to L-HC. * $p < 0.05$, ** $p < 0.001$. The bars indicate mean ± SD.

q = 0.010), in both L-CRC and OB-CRC patients in comparison to L-HC controls. *Victivallis vadensis* (q = 0.012) was significantly higher and *Bacteroides uniformis* (q = 0.010) was significantly lower in L-CRC patients compared to healthy controls. Finally, *Clostridium septicum* (OB-CRC vs. L-CRC, q = 0.025; OB-CRC vs. L-HC, q = 0.004),

Escherichia coli (OB-CRC vs. L-CRC, q = 0.027; OB-CRC vs. L-HC, q = 0.007), *Fusobacterium nucleatum* (OB-CRC vs. L-CRC, q = 0.003; OB-CRC vs. L-HC, q = 0.001), *Enterobacter cloacae* (OB-CRC vs. L-CRC, q = 0.013; OB-CRC vs. L-HC, q = 0.009), and *Streptoccocus bovis* (OB-CRC vs. L-CRC, q = 0.011; OB-CRC vs. L-HC, q = 0.027) were significantly enriched, while *Faecalibacterium prausnitzii* (OB-CRC vs. L-CRC q = 0.011; OB-CRC vs. L-HC q = 0.043) was significantly reduced in OB-CRC patients compared to L-CRC and L-HC subjects.

1.4. SERUM ZONULIN LEVELS

Serum zonulin levels were significantly higher in the OB-CRC group compared to L-HC (26.57 ± 14.95 vs. 14.72 ± 9.57, $p < 0.001$) and L-CRC groups (26.57 ± 14.95 vs. 20.07 ± 15.23, $p = 0.013$). Furthermore, the zonulin levels showed a non-significant trend towards increased concentrations in L-CRC patients compared to L-HC controls (20.07 ± 15.23 vs. 14.72 ± 9.57, $p = 0.804$).

1.5. RELATIONSHIP BETWEEN THE FECAL MICROBIOTA AND SERUM LEVELS OF ZONULIN, TRIMETHYLAMINE N-OXIDE AND INFLAMMATORY MEDIATORS IN THE STUDY GROUPS

Correlation analyses between the abundance of specific bacteria at different taxa levels and serum levels of zonulin, TMAO, and inflammatory mediators (IL-1β and IL-10) in all study groups are shown in Tables 2 and 3.

Subsequent lineal regression analysis showed that the relative abundances of *Ruminococcus* ($R^2 = 0.33$, $\beta = -0.554$, $p = 0.014$) and *Blautia* ($R^2 = 0.33$, $\beta = -0.925$, $p = 0.024$) were negatively associated with zonulin levels in the L-HC control group. Nevertheless, the abundance of *Prevotella* ($R^2 = 0.33$, $\beta = 0.978$, $p = 0.003$) was positively

associated with serum zonulin level in OB-CRC patients, while abundance of *Desulfovibrio* ($R^2 = 0.33$, $\beta = 0.787$, $p = 0.014$) was positively associated to serum zonulin levels in L-CRC patients.

Similarly, regression analysis showed that, in the case of L-HC control subjects, the levels of the anti-inflammatory factor IL-10 were positively associated with the abundance of *Roseburia* ($R^2 = 0.38$, $\beta = 0.681$, $p = 0.001$), while the levels of the inflammatory factor IL-1β were positively associated with the abundance of *Enterobacter* ($R^2 = 0.38$, $\beta = 0.435$, $p = 0.048$). In addition, the level of IL-1β in OB-CRC patients was positively associated with the abundance of *Fusobacterium nucleatum* ($R^2 = 0.38$, $\beta = 0.1963$, $p = 0.050$), while the level of IL-10 was positively associated with the abundance of *Blautia* ($R^2 = 0.38$, $\beta = 0.555$, $p = 0.009$) and *Faecalibacterium prausnitzii* ($R^2 = 0.38$, $\beta = 0.456$, $p = 0.026$). Finally, in the L-CRC group the levels of IL-1β and IL-10 were positively associated with the abundance of *Enterococcus faecalis* ($R^2 = 0.41$, $\beta = 0.418$, $p = 0.037$) and the abundance of *Ruminococcus* ($R^2 = 0.41$, $\beta = 0.418$, $p = 0.022$), respectively.

Table 2. Correlations of gut microbiota composition and serum levels of IL-1B and IL-10 in the L-CRC, OB-CRC, and L-HC groups.

	L-HC	L-CRC	OB-CRC	L-HC	L-CRC	OB-CRC
	IL-1 B			IL-10		
Blautia	−0.621 ($p = 0.024$)	−0.812 ($p = 0.021$)	−0.656 ($p = 0.024$)	0.734 ($p = 0.022$)	0.912 ($p = 0.003$)	0.867 ($p = 0.021$)
Roseburia	−0.625 ($p = 0.032$)	−0.467 ($p = 0.008$)	−0.503 ($p = 0.025$)	0.865 ($p = 0.008$)	0.608 ($p = 0.013$)	0.854 ($p = 0.017$)
Ruminoccocus	−0.745 ($p = 0.038$)	−0.656 ($p = 0.044$)	−0.763 ($p = 0.033$)	0.898 ($p = 0.005$)	0.675 ($p = 0.038$)	0.854 ($p = 0.018$)
Enterobacter	0.843 ($p = 0.015$)	0.827 ($p = 0.017$)	0.834 ($p = 0.015$)	−0.892 ($p = 0.011$)	−0.912 ($p = 0.021$)	−0.895 ($p = 0.012$)
Fusobacterium nucleatum	0.865 ($p = 0.187$)	0.975 ($p = 0.007$)	0.965 ($p = 0.009$)	−0.878 ($p = 0.523$)	−0.997 ($p = 0.003$)	−0.898 ($p = 0.003$)
Streptoccocus	0.721 ($p = 0.211$)	0.815 ($p = 0.0234$)	0.834 ($p = 0.0267$)	−0.754 ($p = 0.323$)	−0.932 ($p = 0.009$)	−0.891 ($p = 0.019$)
Enteroccocus faecalis	0.674 ($p = 0.252$)	0.765 ($p = 0.028$)	0.793 ($p = 0.017$)	−0.763 ($p = 0.237$)	−0.911 ($p = 0.015$)	−0.870 ($p = 0.029$)
Escherichia coli	0.620 ($p = 0.146$)	0.645 ($p = 0.019$)	0.720 ($p = 0.024$)	−0.911 ($p = 0.109$)	−0.867 ($p = 0.012$)	−0.745 ($p = 0.020$)

Table 3. Correlations of gut microbiota composition and serum levels of zonulin and TMAO in the study groups.

	L-HC	L-CRC	OB-CRC		L-CRC	OB-CRC
	Zonulin				TMAO	
Ruminococcus	−0.645 ($p = 0.034$)	−0.523 ($p = 0.031$)	−0.6490 ($p = 0.031$)	*Enterobacteraceae*	0.689 ($p = 0.033$)	0.632 ($p = 0.021$)
Prevotella	0.445 ($p = 0.443$)	0.678 ($p = 0.032$)	0.858 ($p = 0.033$)	*Clostridium*	0.658 ($p = 0.028$)	0.778 ($p = 0.020$)
Blautia	−0.718 ($p = 0.026$)	−0.593 ($p = 0.043$)	−0.631 ($p = 0.049$)	*Streptococcus*	0.631 ($p = 0.049$)	0.593 ($p = 0.043$)
Escherichia coli	0.751 ($p = 0.404$)	0.545 ($p = 0.019$)	0.564 ($p = 0.035$)	*Escherichia coli*	0.763 ($p = 0.021$)	0.790 ($p = 0.019$)
Desulfovibrio	0.578 ($p = 0.367$)	0.748 ($p = 0.035$)	0.804 ($p = 0.031$)	*Desulfovibrio*	0.904 ($p = 0.011$)	0.7489 ($p = 0.038$)

On the other hand, serum levels of TMAO were found to be positively associated with the abundance of *Enterobacteriaceae* (R^2 = 0.43, β = 0.618, *p* = 0.005) and *Escherichia coli* (R^2 = 0.43, β = 0.812, *p* = 0.012) in OB-CRC patients, and with the abundance of *Desulfovibrio* (R^2 = 0.52, β = 0.576, *p* = 0.003) in L-CRC patients. No significant associations were found between any bacterial group and the serum TMAO levels in L-HC controls.

1.6. PREDICTED FUNCTIONAL METAGENOME ANALYSIS

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) was used to identify differences in metagenome functional prediction based on Greengenes 16S rRNA database and Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthologs. The PICRUSt analysis showed that genes involved in energy metabolism (oxidative phosphorylation, q = 0.022), methane and sulfur metabolism (q = 0.015 and q = 0.023, respectively), and glycan biosynthesis and metabolism (lipopolysaccharide biosynthesis, q = 0.05; glycosiltransferases, q = 0.043) were significantly over-represented according to the BMI increase when CRC groups and the L-HC group were compared. Moreover, carbohydrate metabolism (Citrate cycle (TCA cycle), q = 0.032), butanoate metabolism, q = 0.035, and pentose phosphate pathway, q = 0.016), amino acid metabolism (glycine, serine, and threonine metabolism, q = 0.005; valine, leucine, and isoleucine biosynthesis, q = 0.005), metabolism of other amino acids (selenocompound metabolism, q = 0.006) and protein processing in endoplasmic reticulum (q = 0.047) were over-represented in the L-CRC group when compared to the OB-CRC group (**Figure 11**).

Finally, when comparing L-HC controls to both CRC groups we found that gut microbiota from CRC patients was significantly enriched with genes implicated in antigen processing

and presentation (p = 0.004), bacterial chemotaxis (q = 0.013), bacterial secretion system (q = 0.007), and bacterial toxin (q = 0.007) and significantly reduced in genes related to ATP-binding cassette (ABC) transporters (q = 0.022), xenobiotic degradation and metabolism (ethylbenzene degradation q = 0.026) and lipid metabolism functions (fatty acid biosynthesis and degradation (q = 0.017), glycerophopholipid metabolism (q = 0.034), and arachidonic acid metabolism (q = 0.04)) (**Figure 11**).
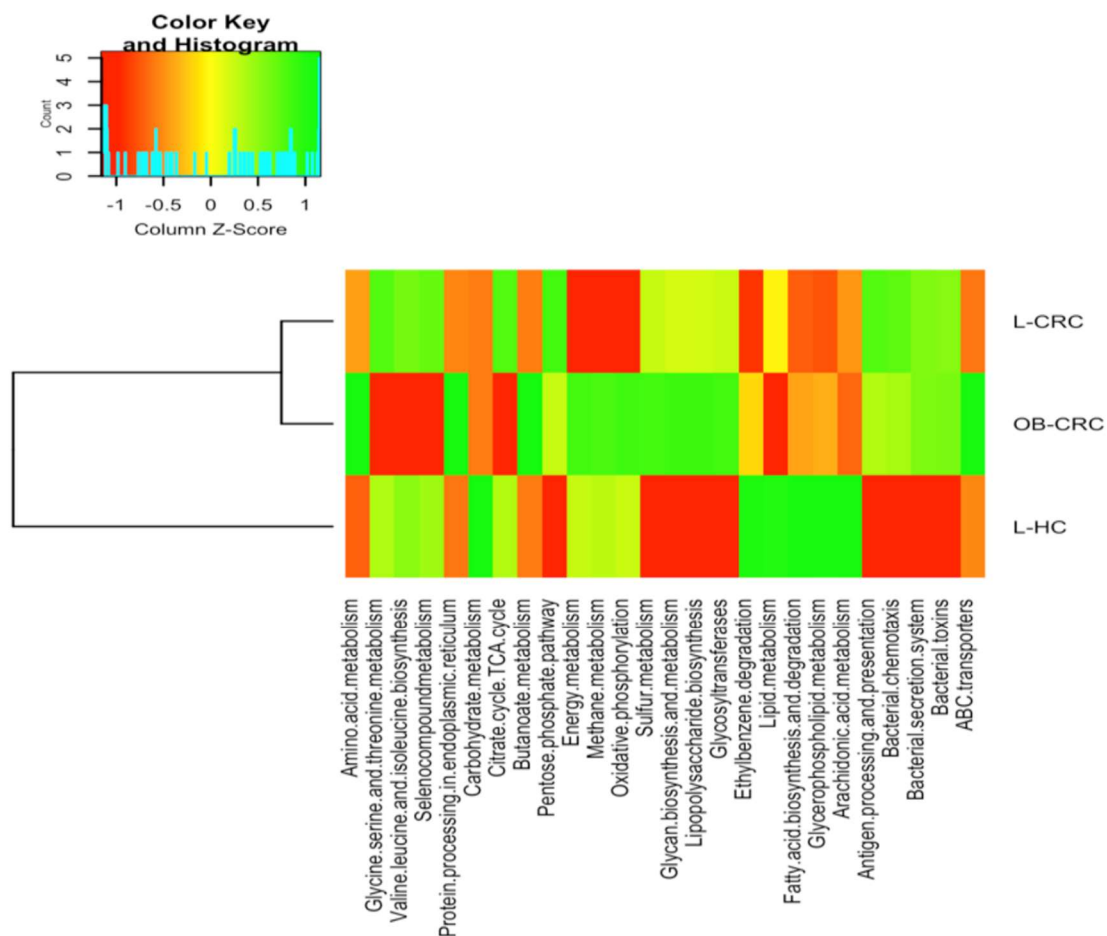
Figure 11. Predicted functional composition of metagenomes based on 16S rRNA gene sequencing data of OB-CRC, L-CRC, and L-HC controls. Heatmap of differentially abundant KEGG pathways identified in the three study groups. The values of color in the heatmap represent the normalized relative abundance of KEGG pathways.

2. RESULTS STUDY 2: Changes in the gut microbiota composition and functionality of colorectal cancer patients after neoadjunvant radiochemotherapy is associated with the pathologic response.

2.1. CLINICAL CHARACTERISTICS OF THE PATIENTS AND HEALTHY CONTROLS

Table 4. Clinical characteristics in the study groups.

	Healthy controls (N=20)	CRC-patients (N=40)	*P	R patients (N=28)	NR patients (N=12)	*P
Age (years)	61.42±7.40	63.35±6.97	0.326	62.93±8.27	63.12±6.34	0.928
Gender, n (M/F)	10 /10	23/17	0.783	16 /12	7/5	0.780
BMI (kg/m^2)	25.45±3.23	26.42±4.71	0.412	26.22±4.22	25.92±3.92	0.835
Constipation, n (%)	6 (20%)	10 (25%)	0.914	7 (25%)	3 (25%)	0.690
Alcohol consumption, n (%)	4 (13.3%)	6 (15%)	0.831	4 (14.28%)	2 (16.16%)	0.740
Current smoking, n (%)	9 (30%)	15 (37.5%)	0.774	11 (39.28%)	4 (33.33%)	0.990
Biochemical data						
Glucose (mg/dl)	94.85±19.86	104.79±27.94	0.161	102.83±26.38	104.15±23.56	0.882
Total cholesterol (mg/dl)	175.2±33.6	183.95±25.71	0.268	184.17±21.64	181.67±26.12	0.755
Triglycerides (mg/dl)	112.67±34.51	114.85±33.62	0.815	109.25±32.12	118.32±27.12	0.398
HDL-cholesterol (mg/dl)	60.7±15.1	54.83±18.23	0.219	55.32±16.21	53.89±18.34	0.807
LDL-cholesterol (mg/dl)	107.78±27.12	112.07±33.45	0.621	109.68±30.29	112.36±33.21	0.805
Histological variables						
Disease stage						
II		22 (55%)	-	15 (53.57%)	7 (58.33%)	0.945
III		18 (45%)	-	13 (46.42%)	5 (41.66%)	0.950
Tumor depth penetration (T)						
T2-T3		26 (65%)	-	18(64.28%)	8 (66.66%)	0.828
T4		14 (35%)	-	10 (35.71%)	4 (33.33%)	0.832
Grade of differentiation						
G1		18 (45%)	-	12 (42.85%)	6 (50%)	0.944
G2		10 (25%)	-	7 (25%)	3 (25%)	0.690
G3		7 (17.5%)	-	5 (18.85%)	2 (16.16%)	0.806
No differentiation		5 (12.5%)	-	3 (10.71%)	2 (16.66%)	0.777

CRC: colorectal cancer; R: responders; NR: non-responders; BMI: body mass index; HDL: high density lipoprotein; LDL: low density lipoprotein. Values are expressed as mean ± SD. *P< 0.05 was considered statistically significant.

CRC patients and healthy controls had comparable eating habits to exclude the influence of dietary differences (data not shown). All CRC patients completed the neoadjuvant radiochemotherapy and underwent surgical resection. There was no significant difference between CRC patients and healthy controls in terms of age, sex, BMI, and biochemical data (**Table 4**). 28 of the 40 CRC patients (70%) had a good response to the neoadju-vant radiochemotherapy (responders, R) (TGR 1-2), whereby 12 (30%) has a poor or non-response (non-responders, NR) (TGR 3-5) to therapy. Both R and NR patients were similar in terms of sex, age, BMI and stage of the cancer, as shown in **Table 4**.

2.2. DIFFERENCES IN TAXNOMIC COMPOSITION AND DIVERSITY OF GUT MICROBIOTA BETWEEN COLORECTAL CANCER PATIENTS AND HEALTHY CONTROLS

A total of 17496823 reads of the 16S rRNA gene (hypervariable V2-V9 regions) were generated from the analyzed stool samples, with an average of 105632 (±10825) reads for each sample, ranging from 359 to 398783. A total of 52844 high quality reads were obtained after trimming and filtering. In the OTUS clustering process a total of 15326 OTUs were obtained, and after alignment of the OTU representative sequences, 2582 OTUs with a relative abundance higher than 1% in at least four samples (97% similarity cut-off) were identified. In the taxonomical assignment process these OTUs were binned in 7 phyla, 39 families, 45 genera and 53 species using QIIME2 pipeline, with Greengenes v13.8 as reference database for sequence classification and alignment.

We first compared the landscape of the gut microbiome in the stool samples of all CRC patients at baseline and in healthy controls in order to define a normal gut microbiota profile. As expected, we found a significant higher diversity and richness (defined by the Shannon and Chao1 indexes, respectively) in the fecal samples of healthy controls with

respect to those of CRC patients (Shannon p = 0.026 and Chao1 p = 0.001) (**Figure 12 A, B**). The beta diversity (Bray-Curtis dissimilarity) comparison of baseline CRC patients and healthy controls indicated that the two cohorts had significantly different genus compositions of intestinal bacteria (p = 0.0001, ANOSIM) (**Figure 12 C**).
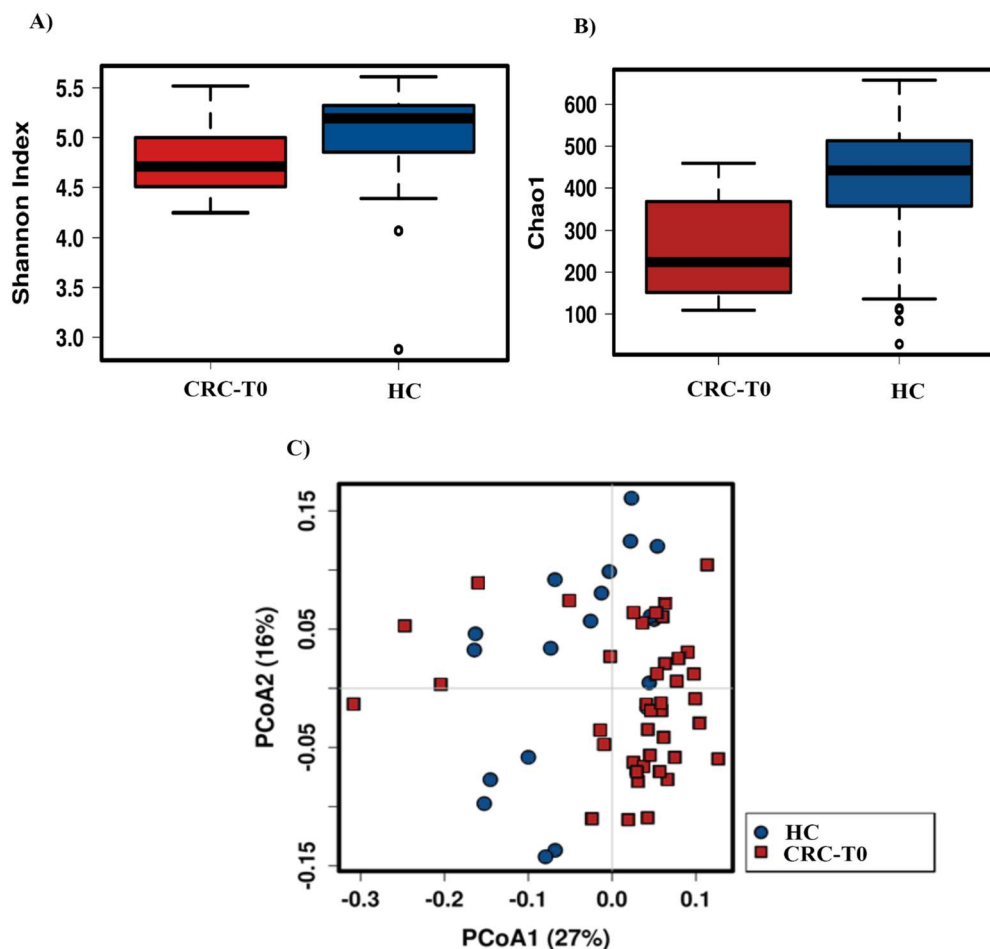
Figure 12. Comparison of alpha and beta diversity among CRC patients at baseline (CRC-T0) and healthy controls (HC). (A) Shannon index (p = 0.026); (B) Chao1 index (p= 0.001). (C) Principal component plot based on Bray-Curtis distance matrix from patients with CRC and healthy controls using Bray-Curtis dissimilarity index al genus-levels (p = 0.0001). The first two coordinates are plotted with the percentage of variability explained indicated on the axis.

Furthermore, the analysis of gut microbiota profiles between CRC patients and healthy controls at baseline revealed significant differences in the abundance at different

taxonomic levels. At phylum level, the relative abundance of Fusobacteria (q < 0.001), Firmicutes (q < 0.001) and Lentisphaerae (q = 0.007) and Proteobacteria (q = 0.003) was increased in patients with CRC, while the relative abundance of Bacteroidetes (q < 0.001) and Actinobacteria (q = 0.034) were significantly decreased in CRC patients when compared to controls (**Figure 13 A**).
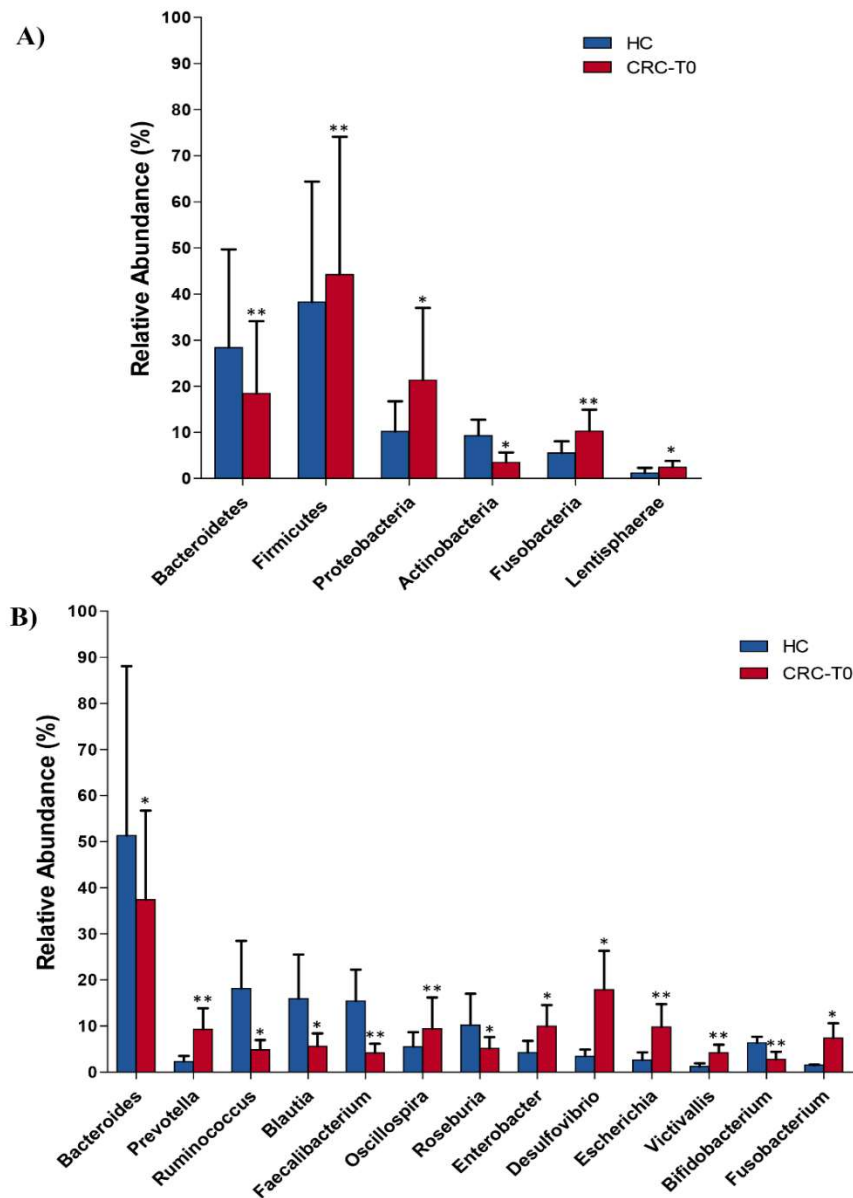
Figure 13. Relative abundance at phylum (A) and genera (B) levels of differentially abundant bacteria in the stool samples of CRC patients at baseline (CRC-T0) and healthy controls (HC). * *p* < 0.05, ** *p* < 0.001**.**

At the genus level, the results indicated significant differences in the microbial composition of dominant genera between CRC patients at baseline and healthy con-trols. Compared with healthy controls, patients with CRC displayed an enrichment in the genera *Prevotella* (q < 0.001), *Oscillospira* (q < 0.001), *Fusobacterium* (q = 0.001), *Enterobacter* (q = 0.020), *Victivallis* (q = 0.012), *Escherichia* (q = 0.046) and *Desulfovibrio* (q < 0.001). Conversely, the abundance of *Bacteroides* (q = 0.003), *Roseburia* (q < 0.001), *Ruminococcus* (q = 0.006), *Faecalibacterium* (q = 0.01), *Bifidobacterium* (q = 0.023) and *Blautia* (q = 0.014) were enriched in healthy controls compared to CRC patients (**Figure 13 B**).

At specie level, while healthy subjects showed a significant higher abundance of *Bifidobacterium bifidum* (q = 0.034) and *Faecalibacterium prausnitzii* (q = 0.040) with respect to CRC patients, *Fusobacterium nucleatum* (q = 0.020), Bacteroides fragilis (q = 0.024) and *Escherichia coli* (q = 0.016) were significantly increased in the fecal samples of CRC patients in comparison to controls.

2.3. CHANGES IN GUT MICROBIOTA DIVERSITY AND COMPOSITION IN RESPONSE TO NEOADJUVANT RADIOCHEMOTHERAPY TREATMENT IN COLORECTAL CANCER PATIENTS

We compared the gut microbiota communities at baseline (T0) versus post-treatment time points (T1, T2 and T3) to study the effect of neoadjuvant RCT on gut microbial diversity and composition in CRC patients. The alpha diversity comparison showed no significant differences in the levels of richness (Chao 1) and diversity (Shannon) between the baseline and the different time points: (Shannon $p = 0.75$ and Chao1 $p = 0.61$) (**Figure 14 A, B**). Moreover, the PCoA plot based on the beta diversity (Bray-Curtis dissimilarity) revealed that the differences of the gut microbial community at T1, T2 and T3 compared

to baseline (T0) were not significant (p = 0.716, ANOSIM) (**Figure 14 C**).
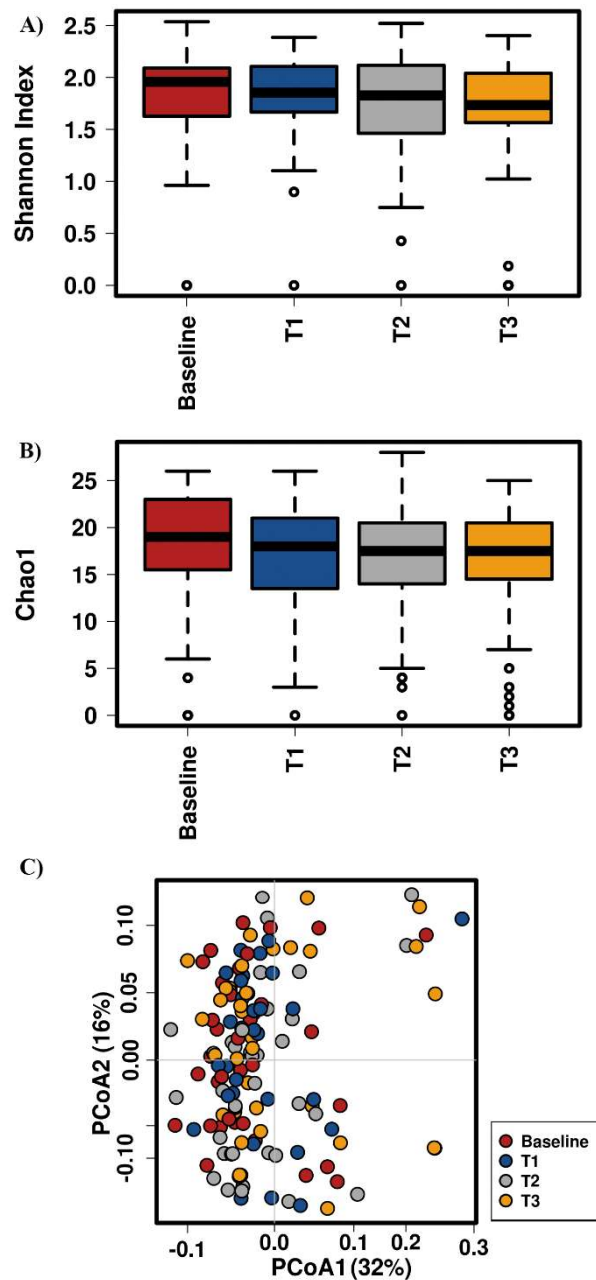
Figure 14. Gut microbiota diversity and richness at baseline and during RTC treatment and post-treatment points in CRC patients. (A) Shannon index (p = 0.75); (B) Chao1 index (p = 0.61); (C) principal component analysis representation based on Bray–Curtis distance matrix of patient distribution based on bacterial genera composition at baseline and during RTC treatment and at post-treatment points (p = 0.716). The first two coordinates are plotted with the percentage of variability, which is explained and indicated on the axis.

The main bacterial phyla (Firmicutes and Bacteroidetes) remained stable over time, while other less abundant phyla such as Fusobacteria and Proteobacteria were significantly decrease at T3 compared to T0 (q = 0.042 and q = 0.039, respectively) in CRC patients. Although bacterial family and genera proportions differed between the different time points, they were not significantly altered by the RCT treatment (Wilcoxon test $p > 0.05$) apart from the genera *Fusobacterium* (q = 0.015), *Escherichia* (q = 0.04) and *Klebsiella* (q = 0.035), which were significantly decreased after treatment, and the genus *Bifidobacterium* (q = 0.049) that was significantly raised at T3 compared to T0 (**Figure 15**).
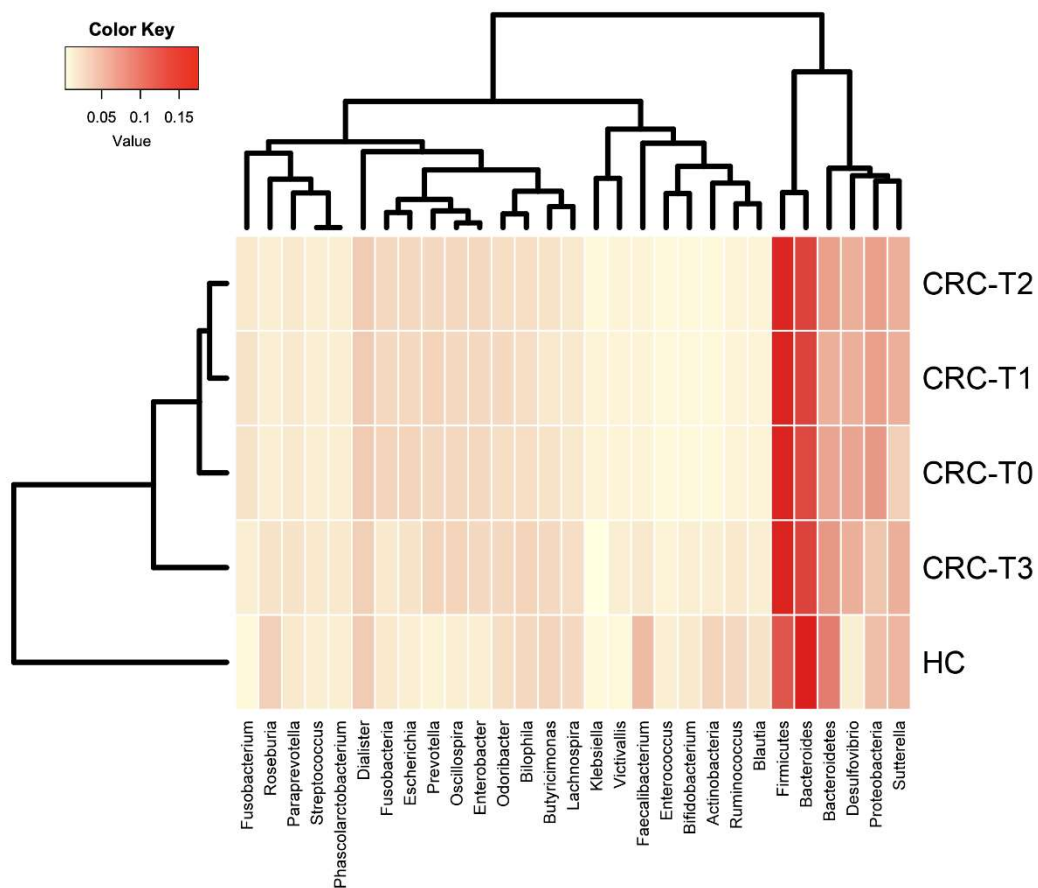
Figure 15. Heatmap diagram of the gut microbiota composition at different taxa levels for baseline (CRC-T0), treatment points with neoadjuvant RCT (CRC-T1, CRC-T2 and CRC-T3), and the healthy control subjects (HC). The 29 phylum and genera that were shared by all of the tested samples (core microbiome) are displayed.

2.4. POST-TREATMENT MICROBIOTA DIVERSITY AND COMPOSITION IS ASSOCIATE TO CLINICAL RESPONSE TO NEOADJUVANT RADIOCHEMOTHERAPY IN CRC PATIENTS

To evaluate the relationship between microbial community and the treatment outcome, we classified the patients based on their response to RCT in categories such as responders (R) and non-responders (NR). As shown in Table 1, no significant differences in terms of stage of cancer, sex, age, and BMI were observed between study groups (R vs. NR).
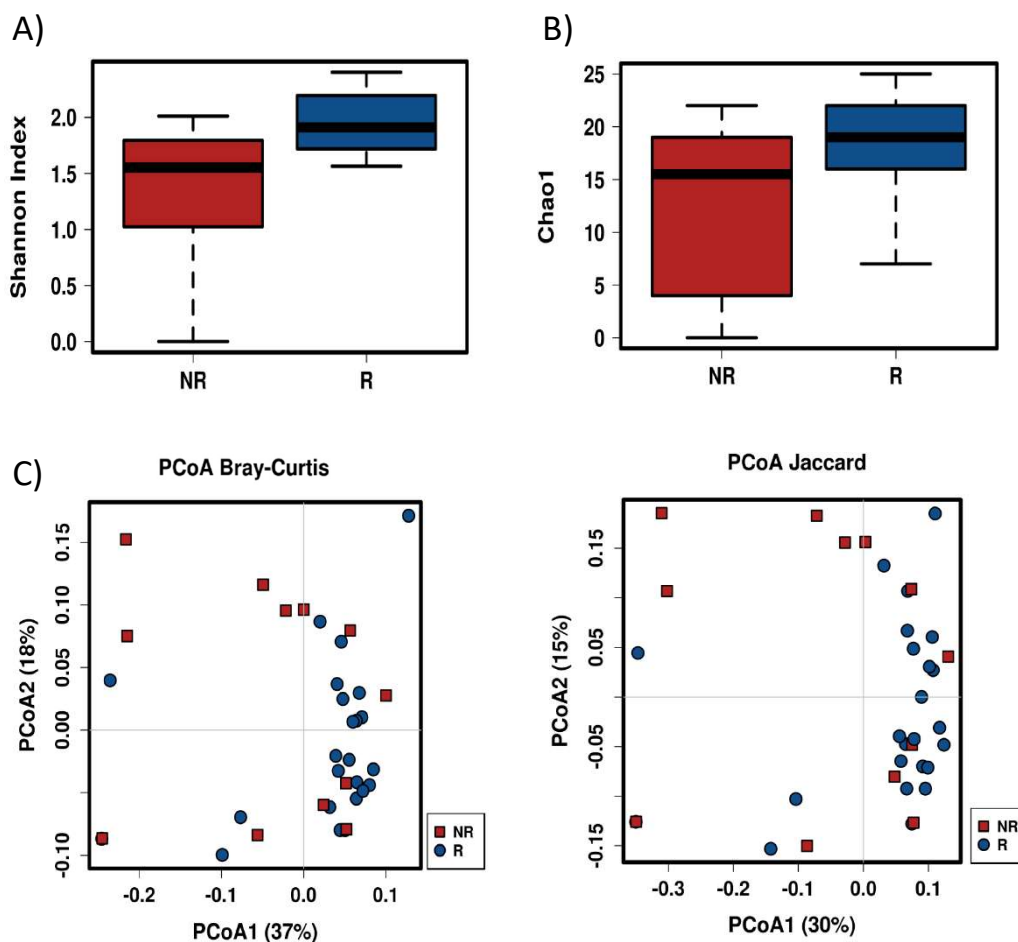
Figure 16. Comparison of alpha and beta diversity in CRC patients according to their response to therapy. (A) Shannon index; (B) Chao1index; (C) principal component plot based on the Bray–Curtis distance matrix and the Jaccard indices from the responder (R) and non-responder (NR) patients at genus-level. The first two coordinates are plotted with the percentage of variability, which is explained and indicated on the axis.

An analysis of alpha diversity at T3 revealed that the R group had higher diversity (Shannon index, q < 0.001; Simpson index, q = 0.039) and richness that the NR group (Chao1 index, q = 0.015) at genus level (**Figure 16 A, B**). Furthermore, the ordination plot based on Bray-Curtis dissimilarities and Jaccard index showed different intestinal microbial compositions at the genus level between both the R and the NR groups at T3 (Bray-Curtis index, q = 0.038; Jaccard index, q = 0.035; non-parametric ANOSIM test) (**Figure 16 C**).

Next, we searched for differentially abundant taxa in the gut microbiome of R versus NR at T3. The analysis revealed that, at the phylum level, there were a significant enrichment in Actinobacteria (q = 0.0025) and Firmicutes (q = 0.0017) populations and a significant decrease in Fusobacterias (q = 0.025) and Proteobacterias (q = 0.037) populations in the R group in comparison to the NR group (**Figure 17 A, B**). At the family level, a significant higher abundance of Ruminococcaceae (q = 0.004) and Bifidobacteriaceae (q = 0.03), accompanied by a significant lower abundance of Prevotellaceae (q = 0.045), Enterobactericeae (q = 0.027) and Fusobacteriaceae (q = 0.014) were showed in the R group compared to NR group (**Figure 17 C**).

In addition, at the genera level, we identified a significant increase in *Ruminococcus* (q = 0.035), *Bilophila* (q = 0.008), *Collinsiella* (q = 0.015), *Bifidobacterium* (q = 0.024), *Roseburia* (q = 0.032), and *Faecalibacterium* (q = 0.041) in R patients with respect to NR, while a significant increase in *Prevotella* (q = 0.05), *Fusobacterium* (q = 0.045), *Escherichia* (q = 0.037), *Bacteroides* (q = 0.027) and *Klebsiella* (q = 0.035) were observed in NR patients compared to the R group (**Figure 18 A, B**). Finally, at the specie level, we found a significant overabundance of *Prevotella copri* (q < 0.001), *Escherichia coli* (q = 0.029), *Fusobacterium nucleatum* (q = 0.015) and *Bacteroides fragilis* (q = 0.029) in the NR group, while the R group displayed a significantly higher abundance of

Bifidobacterium bifidum (q = 0.043), *Ruminococcus albus* (q= 0.019), *Collinsella aerofaciens* (q = 0.018) and *Faecalibacterium prausnitzii* (q = 0.027).
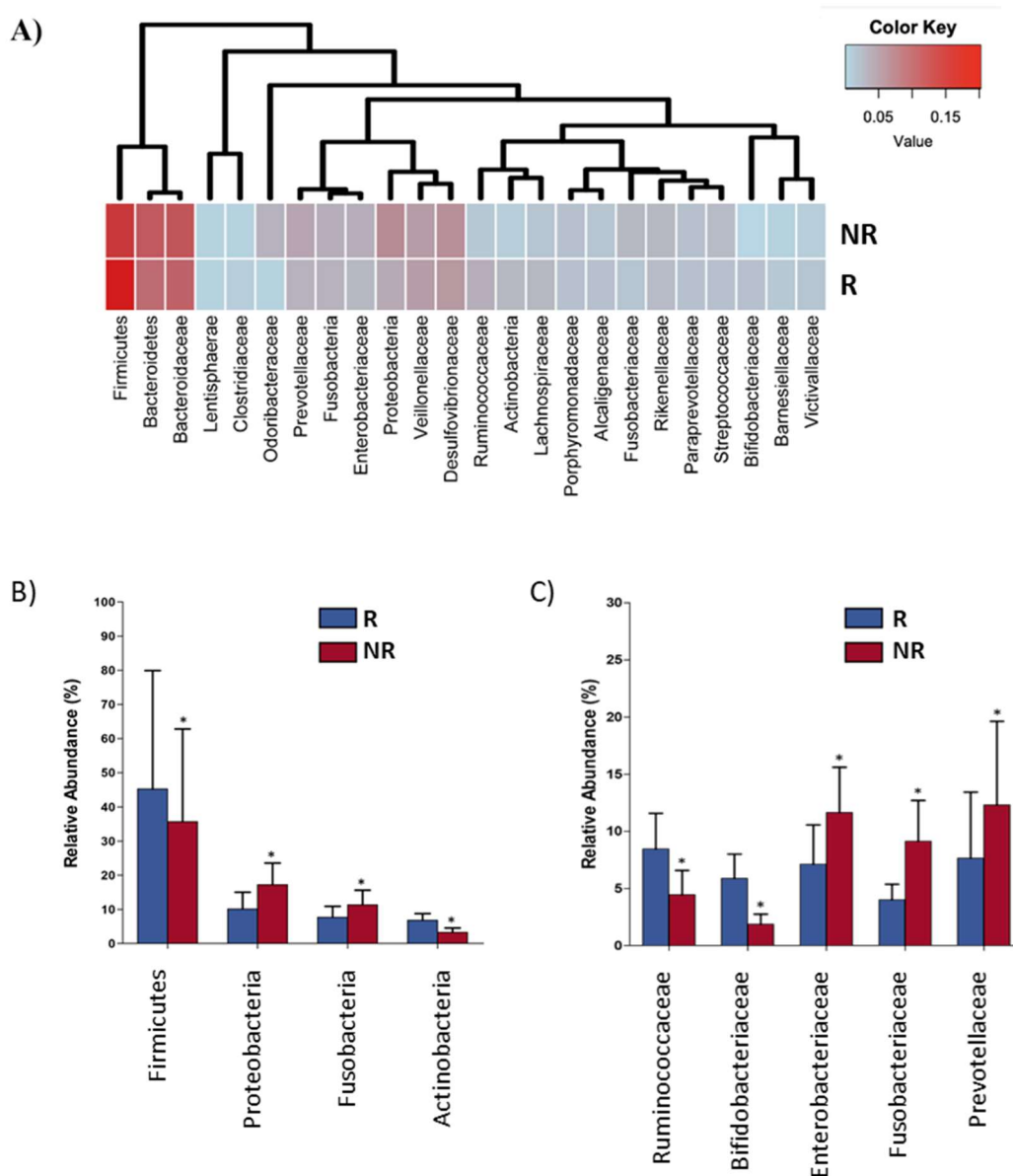
Figure 17. Heatmap of the fecal microbiota composition at the phylum and family levels in the responder (R) and non-responder (NR) patients (A). Relative abundance at phylum (B) and family (C) levels of differentially abundant OTUs in the stool samples of N patients compared to the NR patients. * p < 0.05.
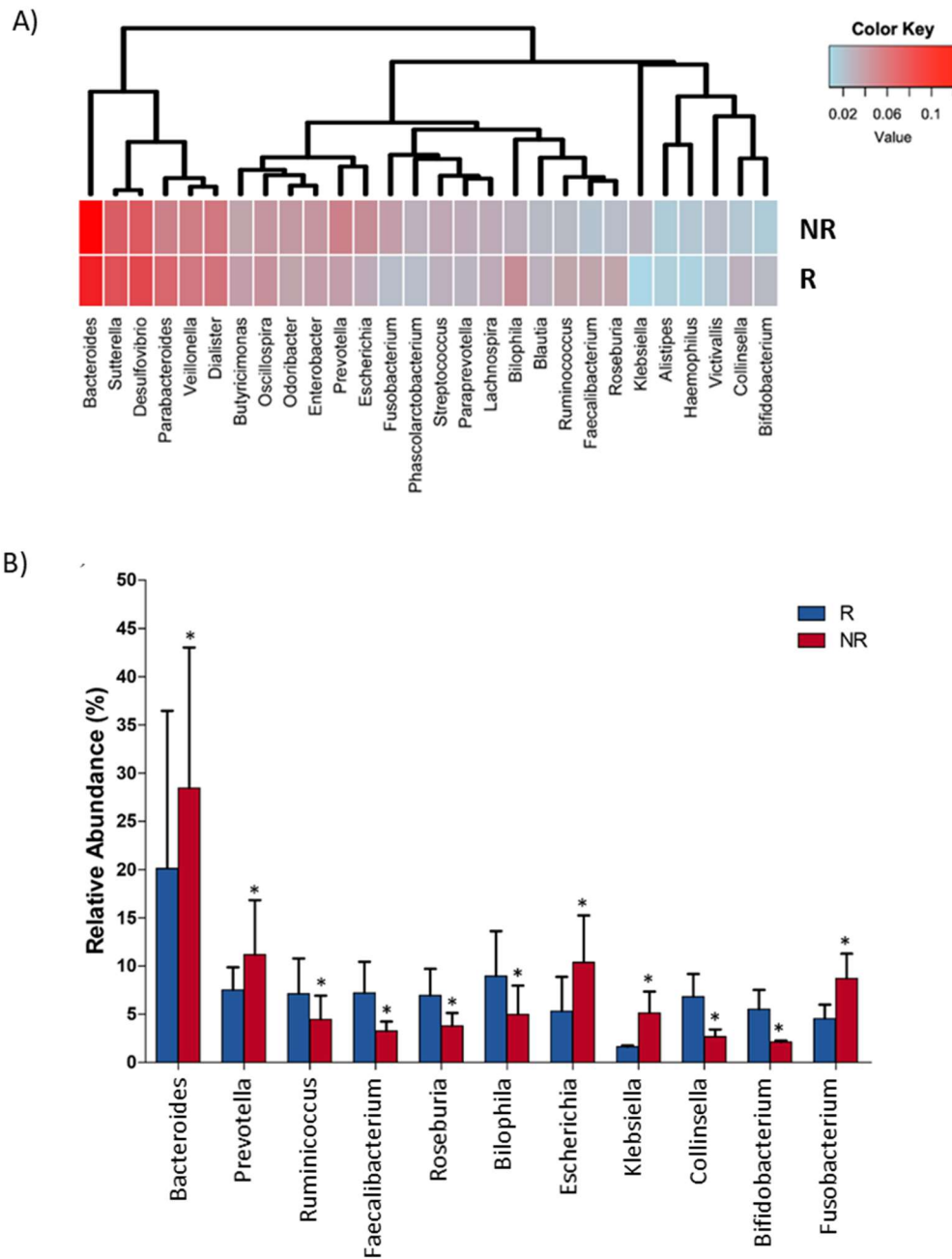
Figure 18. Heatmap of the fecal microbiota composition at genera level in the responder (R) and non-responder (NR) patients (A). Relative abundance at genera level of differentially abundant OTUs in the stool samples of the N patients compared to the NR patients. * p < 0.05 (B).

2.5. BASELINE MICROBIOTA COMPOSITION COULD PREDICT RESPONSE TO RADIOCHEMOTHERAPY TREATMENT IN COLORECTAL CANCER PATIENTS

After described significant differences in intestinal microbial composition be-tween R and NR after RCT treatment, we next assessed the predictive power of gut microbiome related to neoadjuvant RCT response. We used Random (RF) to build a predictive model based on the overall gut microbiota profile using the species-level abundance data as input. After RF analysis with 500 bootstrap samples, we found that overall gut microbiota composition data had a significant accuracy of 80% and area under the curve (AUC) of 0.71. The main species accounting for this stratification were *Ruminococcus albus*, *Bifidobacterium bifidum*, *Faecalibacterium prausnitzii*, *Fusobacterium nucleatum*, and *Bacteroides fragilis*, and when proportions of these bacterial species were only used for testing the accuracy of the RF classifier this increased to 96% (AUC = 0.925). Thus, the response to RTC or the lack of it were identified with an accuracy of 94% (AUC = 0.95) and of 91% (AUC = 0.92), respectively (**Figure 19 A**). The validation cohort consisted of 84 CRC patients under neoadjuvant RCT (45 R patients and 39 NR patients) (data collected from the Genome Sequence Archive in National Genomics Data Center, accession number CRA002850). After RF analysis in this validation cohort, an accuracy of 92.0% (AUC = 0.93) and 90.0% (AUC = 0.91) were obtained for the response to RTC or the lack of it, respectively **(Figure 19 B)**. Among the five species variables, *Fusobacterium nucleatum*, and *Bacteroides fragilis* were biomarkers of R patients, and *Ruminococcus albus*, *Bifidobacterium bifidum*, and *Faecalibacterium prausnitzii* were biomarkers of NR patients.
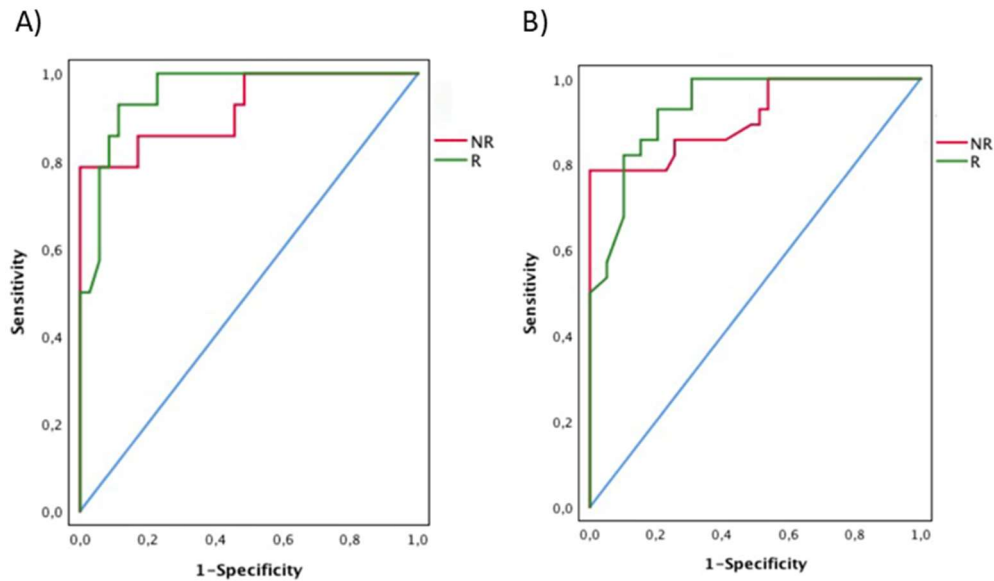
Figure 19. Receiver operating characteristic (ROC) curve based on the random forest classifier constructed using microbial variables (*Ruminococcus albus*, *Bifidobacterium bifidum*, *Faecalibacterium prausnitzii*, *Fusobacterium nucleatum*, and *Bacteroides fragilis*). (A) Training cohort. The area under the ROC curve (AUC) was 0.95, and the 95% confidence interval (CI) was 0.901–1 for the R patients (green), and the AUG was 0.92 and 95% the IC was 0.827–1 for the NR patients (red). (B) Validation cohort. The AUG was 0.93 and the 95% IC was 0.877–0.987 for the R patients (green), and the AUG was 0.91 and 95% the IC was 0.835–0.984 for the NR patients (red).

2.6. DIFFERENCES IN THE GUT MICROBIOTA FUNCTIONS BETWEEN RESPONDER AND NON-RESPONDER

KEGG pathway enrichment analysis of the metagenomic data showed that genes for energy metabolism such as methane metabolism ($q < 0.004$), carbohydrate metabolism such as pentose phosphate pathway ($q = 0.0022$), pyruvate metabolism ($q < 0.001$), starch and sucrose metabolism ($q = 0.008$), galactose metabolism ($q = 0.007$), butanoate metabolism ($q = 0.005$) and glycolysis-gluconeogenessis ($q = 0.0028$) and xenobiotics biodegradation and metabolism pathways including benzoate degradation ($q = 0.038$) and nitrotoluene degradation ($q = 0.005$) and membrane transport such as ABC transporters

(q = 0.012) and transporters (q = 0.012) were significantly depleted in NR compared to R patients.
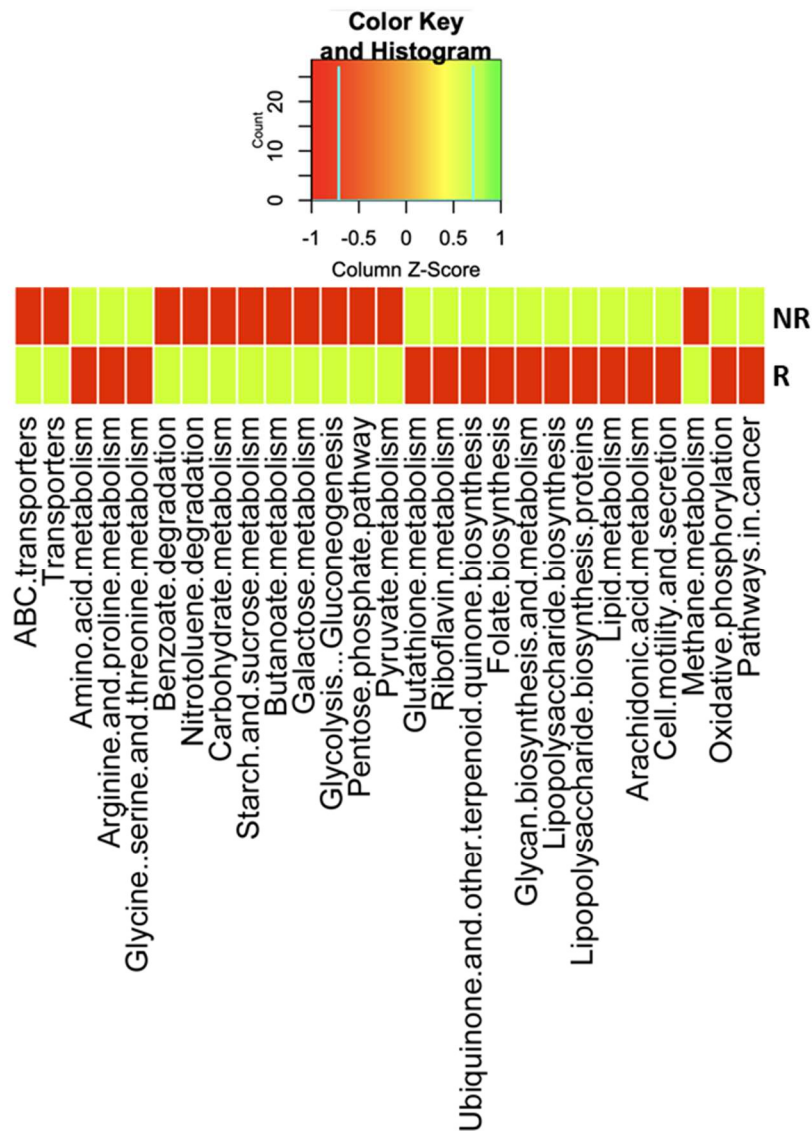
Figure 20. Heatmap of bacterial gene functional predictions using the PICRUSt algorithm from the fecal samples from the responder (R) patients and the non-responder (NR) patients.

Nevertheless, NR patients compared to R, there was a significant over-representation of genes for the lipid metabolism such as araquidonic acid metabolism (q = 0.006), amino acid metabolism pathways, such as for arginine and proline metabolism (q = 0.029) and glycine, serine and threonine metabolism (q = 0.001), as well as in genes for metabolism

of other amino acids such as glutathione metabolism (q = 0.003), metabolism of cofactors and vitamins such as riboflavin metabolism (q = 0.003), ubiquinone and other terpenoid metabolism (q < 0.001) and folate biosynthesis (q = 0.014), glycan biosynthesis and metabolism such as lipopolysaccharide biosynthesis (q = 0.007) and lipopolysaccharide biosynthesis proteins (q = 0.001), cellular processes and signaling that contains cell motility and secretion (q = 0.0018) and oxidative phosphorylation (q < 0.001) and pathways in cancer (q < 0.001) (**Figure 20**).

2.7. CHANGES IN SERUM LEVEL OF POLYAMINES AND ZONULIN AND FECAL LEVEL OF SHORT CHAIN FATTY ACIDS AFTER RADIOCHEMOTHERAPYTREATMENT IN COLORECTAL CANCER PATIENTS

Significant differences in the serum levels of several polyamines and acetyl derivatives of polyamines were found in R and NR patients at post-treatment point (T3). Then, we have found in NR a significant increase in the levels of spermine, N1-acetyl spermine (N1-AcSP), N1, N12-diacetylspermine (N1,N12-DiAcSP), N1-acetylspermidine (N1-AcSPD), N1, N8- diacetylspermidine (N1,N8-DiAcSPD) and N1-acetylputrescine (N1-AcPUT) compared to R patients. On the other hand, within-group there were also significant changes in the levels of N1-AcSPD and spermine in both R and NR patients and in the serum levels of N8-AcSPD only in the NR group (**Table 5**).

Table 5. Serum polyamine and zonulin levels in baseline (T0) and post-treatment (T3).

	R (N=28)	NR (N=12)	Between-group Difference[1]	P[2]
Agmatine (ng/mL)				
Baseline	0.11±0.13	0.13±0.15		
Post-treatment	0.25±0.24	0.17±0.15	-0.025 (-0.11, 0.63)	0.571
Change	0.14 (-0.27, -0.13)	0.035 (-0.13, 0.061)		
Arginine (µg/mL)				
Baseline	23.18±4.20	24.54±4.76		
Post-treatment	22.82±4.16	23.10±4.48	-1.35 (-4.05, 1.35)	0.319
Change	-0.36 (-1.5, 2.27)	-1.43 (-1.13, 4.0)		
Ornithine (µg/mL)				
Baseline	19.46±5.74	23.31±8.06		
Post-treatment	20.21±4.16	22.80±7.55	-3.85 (-8.07, 0,37)	0.073
Change	0.74 (-3.69, 2.19)	-0.51 (-3.72, 4.74)		
N1,N12-DiAcSP (ng/mL)				
Baseline	1.08±0.43	1.68±1.34		
Post-treatment	0.90±0.52	1.22±0.57	-0.59 (-1.20, 0.06)	0.015
Change	-0.18 (0.017, 0.34)	0.46 (-0.152, 1.07)		
N1,N8-DiAcSPD (ng/mL)				
Baseline	0.71±0.26	0.99±1.03		
Post-treatment	0.74±0.34	0.88±0.38	-0.28 (-0.74, 0.17)	0.007
Change	0.03 (-0.13, 0.059)	-0.11 (-0.34, 0.57)		
N1-AcSPD (ng/mL)				
Baseline	22.47±7.10	27.68±13.47		
Post-treatment	23.42±8.26	28.89±10.38	-5.21 (-11.73, 1.3)	0.021
Change	0.94 (-3.88, 1.99)*	1.20 (-6.10, 3.68)*		
N8-AcSPD (ng/mL)				
Baseline	14.52±3.48	14.88±3.27		
Post-treatment	14.69±3.39	16.10±2.33	-0.35 (-2.38, 1.67)	0.727
Change	0.16 (-0.90, 0.57)	1.22 (-2.42, -0.20)*		
N-AcPUT (ng/mL)				
Baseline	5.04±1.60	5.92±5.38		
Post-treatment	5.39±3.79	4.77±1.70	-0.88 (-3.29, 1.53)	0.030
Change	0.34 (-1.78, 1.09)	-1.15 (-1.01, 3.32)		
Putrescine (ng/mL)				
Baseline	8.84±4.40	7.95±3.52		
Post-treatment	8.06±3.89	7.47±3.09	0.89 (-1.49, 3.28)	0.457
Change	-0.78 (-0.39, 1.96)	-0.47 (-1.07, 2.02)		
Spermidine (ng/mL)				
Baseline	17.14±7.19	22.26±12.69		
Post-treatment	20.42±12.40	20.90±10.81	-5.11 (-11.36, 1.12)	0.106
Change	3.28 (-7.42, 0.85)	-1.35 (-2.01, 4.73)		
N1-AcSP (ng/mL)				

Baseline	0.89±0.33	1.48±0.70		
Post-treatment	1.19±0.63	1.33±0.62	-0.58 (-0.92, -0.25)	0.014
Change	0.29 (-0.55, -0.046)	-0.14 (-0.11, 0.40)		
Spermine (ng/mL)				
Baseline	3.77±1.30	12.10±7.85		
Post-treatment	4.80±2.88	7.35±3.66	-8.32 (-11.74, -4,89)	0.001
Change	1.03 (-2.17, 0.107)*	-4.74 (1.71, 7.77)*		
Zonulin (ng/mL)				
Baseline	257.6±65.4	272.6±35.1		
Post-treatment	218.1±76.4	298.4±47.5	-22.2 (7.6, -10.2)	0.004
Change	-39.3 (-29.2, 23.9)	25.2 (11.3, 37.1)		

Serum polyamine levels were measured by means of ultra-high performance liquid chromatography tandem mass spectrometry (UHPLCMS/MS). Values are expressed as mean ± SD for baseline and post-treatment values or mean (95% CI) for the mean differences between baseline and post-treament timepoint T3. R: responder; NR: non-responder. [1] Difference between R and NR patients at post-treatment when adjusted for baseline. [2] Comparison among post-treatment changes was conducted with a covariance model (ANCOVA) adjusted for baseline. * Wilcoxon signed-rank test was used to calculate differences in polyamines between baseline and post-treatment in R and NR patients. $p < 0.05$ was considered statistically significant.

SCFAs are bacterial-derived metabolites with important physiological functions in the host and with anti-cancer properties. Analysis of the fecal levels of SCFAs revealed significant differences in the concentrations of acetic, butyric, isobutyric, valeric, isovaleric and hexanoic acids between the R and NR study groups at post-treatment time point T3. Moreover, we found several significant differences in the within-group comparison of the fecal concentrations of acetic and butyric acids, which significatively increased after RCT treatment in the R group. On the other hand, serum zonulin levels (a circulating marker of gut permeability) were significantly increased in the NR group (but not in R group) after RCT treatment (**Table 6**).

Table 6. Fecal SCFAs concentrations in Baseline (T0) and post-treatment (T3).

	R (N=28)	NR (N=12)	Between-group Difference1	P^2
Acetic acid				
Baseline	0.83±0.39	0.71±0.15	0.26 (-0.03, 0.56)	0.012
Post-treatment	1.04±0.40	0.77±0.17		
Change	0.20 (-0.39, -0.007)*	0.06 (-0.30, 0.18)		
Propionic acid				
Baseline	1.40±1.27	2.02±1.35	0.79 (-0.16, 1.76)	0.102
Post-treatment	1.50±1.10	0.70±1.52		
Change	0.09 (-0.59, 0.79)	-1.31 (-2.9, 0.36)		
Butyric acid	0.93±0.68	1.37±0.45	1.33 (-0.04, 2.71)	0.016
Baseline	2.36±1.82	1.02±1.07		
Post-trearment	1.43 (-2.15, -0.70)*	-0.344 (-0.65, 1.34)		
Change				
Isobutyric acid	0.31±0.33	0.58±0.33	0.15 (0.03, 0.26)	0.010
Baseline	0.24±0.15	0.09±0.05		
Post-trearment	-0.07 (-0.07, 0.21)	-0.49 (0.23, 0.76)		
Change				
Valeric acid				
Baseline	0.30±0.16	0.61±0.32	0.15 (0.005, 0.29)	0.002
Post-trearment	0.29±0.19	0.13±0.07		
Change	-0.01 (-0.07, 0.09)	-0.47 (0.18, 0.76)		
Isovaleric acid				
Baseline	0.50±0.49	0.90±0.44	0.18 (0.05, 0.29)	0.009
Post-trearment	0.39±0.24	0.20±0.13		
Change	-0.11 (-0.09, 0.31)	-0.69 (0.36, 1.02)		
4-methylvaleric acid				
Baseline	0.13±0.23	0.37±0.64	0.02 (-0.05, 0.10)	0.216
Post-treatment	0.07±0.10	0.04±0.01		
Change	-0.06 (-0.02, 0.15)	-0.33 (-0.20, 0.86)		
Hexanoic acid				
Baseline	0.15±0.20	0.11±0.08	0.05 (-0.19, 0.13)	0.007
Post-trearment	0.10±0.10	0.05±0.09		
Change	-0.04 (-0.01, 0.10)	-0.05 (-0.01, 0.13)		
Heptanoic acid				
Baseline	0.09±0.15	0.07±0.06	0.02 (-0.007, 0.04)	0.171
Post-trearment	0.06±0.06	0.05±0.01		
Change	-0.02 (-0.01, 0.07)	-0.028 (-0.02, 0.08)		

Short-chain fatty acids (SCFAs) in fecal samples were analyzed by means of gas chromatography coupled with a flame-ionization detector (GC-FID). Values are expressed as mean ± SD for baseline and post-treatment values or mean (95% CI) for the mean differences between baseline and post-treament timepoint T3. R: responder; NR: non-responder. 1 Difference between R and NR patients at post-treatment when adjusted for baseline. 2 Comparison among post-treatment changes was conducted with a covariance

model (ANCOVA) adjusted for baseline. * Wilcoxon signed-rank test was used to calculate differences in the SCFAs and zonulin between the baseline and post-treatment in R and NR patients. p < 0.05 was considered statistically significant.

In addition, pairwise comparisons using Spearman rank correlation analysis were then performed between bacterial species enriched in the gut microbiome of both R and NR patients and the fecal SCFAs and serum zonulin and polyamines levels. Interestingly, we found a statistically significant positive correlation between the fecal levels of butyrate and the abundance of the *Faecalibacterium prausnitzii* (r = 0.816; p < 0.001) and *Ruminoccocus albus* (r = 0.924; p = 0.008) in the R group and between the concentration of propionic acid and *Bacteroides fragilis* in the NR group.

In addition, negative associations of *Faecalibacterium prausnitzii* with the serum levels of spermine (r = -0.619; p = 0.018) and N,N12-DiAcSP (r = -0.793; p = 0.01) in the R patients were described, while there was a positive association between de abundance of *Bacteroides fragilis* and *Fusobacterium nucletum* with the levels of N,N12-DiAcSP (r = 0.436; p = 0.043; r = 0.637; p = 0.001, respectively) and N8-AcSPD (r = 0.547; p = 0.014; r = 0.752; p < 0.001) in the NR patients. Finally, *Prevotella copri* was positively associated to the serum zonulin levels in the NR patients.

DISCUSSIONS

In these studies, we showed that the composition of the gut microbiome from CRC patients (both obese and non-obese) was significantly different to the gut eubiotic microbiota of non-obese healthy subjects. Moreover, we found an obesity-related microbial profile linked to CRC, that could be responsible for the significantly higher serum levels of zonulin (marker of intestinal permeability), TMAO (cardio-vascular-diseases-related microbial metabolite), and IL-1β (pro-inflammatory factor) and the lower levels of IL-10 (anti-inflammatory factor) compared to non-obese CRC patients and controls.

Loss of microbial diversity has been associated with chronic health conditions [291, 292] and cancer [293, 294], and with poor outcomes to certain forms of cancer therapy [295-297]. Accordingly, recent works have also reported that patients with CRC display a lower bacterial diversity and richness in fecal samples and intestinal mucosa compared to healthy individuals [126, 127]. We found that compared to healthy controls, the CRC microbiota exhibited a state of dysbiosis with a reduced overall bacterial richness and diversity.

Moreover, the analysis of the alpha diversity (community composition) of the gut microbiome from the OB-CRC, L-CRC and L-HC controls revealed a decreased richness (Chao1 index) and diversity (Shannon index) in the OB-CRC and L-CRC groups compared to L-HC controls. Nevertheless, no significant differences in Chao1 and Shannon indices were found between OB-CRC and L-CRC groups. These results may suggest that the decrease in gut microbiota diversity of CRC patients could not be entirely related to a history of obesity. In addition, the Bray–Curtis dissimilarity plot analysis to detect microbial community differences in structure clustered OBC-CRC and L-CRC patients together, but clustered L-HC controls separately, confirming that obesity does

not introduce important changes to the overall structure of the gut microbial community in CRC patients. In this regard, a recent meta-analysis done by Greathouse *et al*. described no universal differences in alpha and beta diversity between obese and non-obese patients with CRC, suggesting that, similarly to the community composition, community structure is not associated with BMI in CRC patients [231].

Furthermore, we have showed that CRC patients exhibit clear differences in gut microbiota composition when compared to healthy individuals, independently of the BMI of the patient. On one hand we identified an increase of Firmicutes, Fusobacteria, and Proteobacteria phyla in fecal samples from CRC patients. Remarkably, these phyla have been previously associated with gut dysbiosis, inflammation, and CRC [298]. On the other hand, genus-level analyses confirmed that the intestinal microbiota of CRC patients with or without obesity is characterized by a reduction of butyrate-producing bacteria and an increase in harmful bacterial species that could act as opportunist pathogens with pro-inflammatory and pro-carcinogenic properties. Accordingly, other works have shown that CRC patients display an enrichment in pro-inflammatory opportunistic pathogens and a decrease in butyrate-producing bacteria, which may lead to an imbalance in intestinal homeostasis (dysbiosis) that could ultimately lead to tumor formation [136, 299]. These CRC-related significant alterations in specific bacterial groups suggest that microbial dysbiosis was already present in CRC at the time of diagnosis and have been proposed to have a potential impact on mucosal immune response [127].

Nevertheless, we found a significant increase in the abundance of several specific taxa of opportunist pathogens in the gut microbiome of OB-CRC patients compared to L-CRC and L-HC subjects. In particular, in the obese CRC group we detected a significant rise in the abundance of the families Enterobacteraceae and Streptococcaceae and the genera/species *Enterobacter* (*E. cloacae*), *Escherichia* (*E. coli*), and *Streptococcus* (*S.*

bovis). Enterobacteriaceae are normal commensal bacteria in the human gut. However, the family includes numerous genera of bacteria that are potentially pathogenic, such as *Salmonella*, *Shigella*, *Escherichia*, *Enterobacter*, *Proteus*, and *Klebsiella* [300]. Previous studies have reported that Enterobacteriaceae is more abundant in patients with inflammatory bowel disease or CRC in comparison to healthy individuals [129].

Gut microbiota might directly influence the relationship between obesity and CRC. In this study, we found that OB-CRC patients have significantly higher plasma levels of TMAO when compared with L-CRC and L-HC subjects. Barrea *et al*. demonstrated that circulating levels of TMAO increased along with BMI in patients with overweight or obesity [301]. Another recent study also reported increased serum TMAO levels among CRC patients, compared to healthy controls, rendering TMAO as a potential prognostic marker for CRC [226]. Additionally, we found that the presence of certain specific bacterial taxa in human feces of both CRC groups were associated with the concentration of plasma TMAO. We observed that the serum TMAO concentrations were significantly and positively associated with the abundance of the family Enterobacteriaceae and species *Escherichia coli* in OB-CRC patients and the abundance of *Desulfovibrio* in L-CRC patients. In agreement with our results, other human and animal studies have suggested that several families of bacteria are involved in the production of TMA/TMAO such as *Prevotellaceae* [302] and *Enterobacteriaceae* [303, 304]. Moreover, a novel microbial, the cntA/B, has been found to be able to convert carnitine into TMA and this gene was reported to exist among only few species including *Escherichia coli*, *Klebsiella* spp., and *Citrobacter* spp. [305]. Additionally, it has been previously described that the increase in the conversion of choline to TMA can be caused also by the expression of the cutC gene by bacteria such as *Desulfovibrio* [303]. Then, the increase of specific pathogenic bacteria such as *Escherichia coli* in OB-CRC patients can be responsible (at

least partially) for the significant increase in microbial-derived proinflammatory molecules such as TMAO.

Nevertheless, blood TMAO levels not only depend on the gut microbiota composition and metabolic activities [306], but also on the functioning of the gut–blood barrier that controls the access of gut-derived molecules to the bloodstream [307]. Accordingly, we found that plasma zonulin levels were significantly higher in the OB-CRC patients compared to L-CRC and L-HC controls. Increased zonulin level was associated with the abundance of *Prevotella* in OB-CRC patients. *Prevotella* contains enzymes that are important in mucin degradation, which may disrupt the colonic mucus barrier and impair the intestinal barrier function [308], and therefore may contribute to increase the circulating levels of TMAO. Recent evidence has suggested that TMAO could play a role in the inflammatory process and that this inflammation induction could be a possible factor that provides a link between TMAO and cancer. Serum level of TMAO was found to synergize the pro-inflammatory effects of *Helicobacter* pylori infections on gastric epithelial cells, through the increase in the expression level of pro-inflammatory genes such as IL-6 and chemokine ligands [309], which play roles in cancer progression [310]. In another study, Yue *et al*. also demonstrated that TMAO can trigger the activation of the NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome [227], which has been suggested to be implicated in the growth and/or metastasis of a variety of cancers including CRC [311]. Nevertheless, further research is necessary to specify the mechanism by which TMAO is linked to CRC via inflammation induction.

With respect to the obesity-specific microbiota observed in in OB-CRC patients, the passenger *Fusobacterium nucleatum* has been reported to be more abundant in people who are obese than in lean people [312]. *Fusobacterium nucleatum* is an opportunistic pathogen closely associated with the occurrence and development of periodontitis, whose

relationship with CRC has been widely reported [151, 313, 314]. We found an association of this species with the higher levels of the proinflammatory IL-1β in OB-CRC patients. Increased abundance of *Fusobacterium nucleatum* has been previously associated to a higher expression of pro-inflammatory cytokines in colonic tissue from CRC patients [153, 154]. Thus, Kostic *et al*. suggested that *Fusobacterium nucleatum* induced a NF-κB-driven proinflammatory response to promote CRC [315]. In addition, Rubinstein *et al*. described that *Fusobacterium* spp. function includes the induction of inflammatory responses and colon tumor cell growth promotion via β-catenin activation [115]. Furthermore, IL-1β induces the activation of the Wnt signaling pathway by phosphorylation of Glycogen synthase kinase 3 beta (GSK3β) [316], a key signaling pathway for intestinal tumorigenesis [317], supporting the central role of IL-1β in CRC pathogenesis

On the other hand, previous studies have described that IL-10 deficiency leads to elevated levels of TNF-α, IL-6, and IL-17, triggering chronic inflammation and promoting tumor growth [318]. In this study, the lowest levels of anti-inflammatory IL-10 found in the OB-CRC patients were associated to the lowest abundance of *Blautia* and *Faecalibacterium prausnitzii*. All these bacteria are important suppliers of butyrate to the colonic epithelium. Butyrate is a SCFA considered as the most important nutrient for epithelial cells of the colon and has a role in the epigenetic control of gene expression, while also functioning as a mediator of anti-inflammatory responses, the maintenance of the intestinal barrier integrity, and the protection against oxidative stress [289, 319]. Therefore, butyrate promotes the integrity of gut epithelial tight junctions as well as increases the release of the anti-inflammatory cytokine IL-10 [320], that protects against cancer formation.

Finally, our PICRUSt analysis suggests a lower relative abundance of genes responsible for carbohydrate metabolism functions such as butanoate metabolism and pentose phosphate pathway, together with genes for the amino acid metabolism and protein processing in endoplasmic reticulum were found depleted in OB-CRC patients compared to L-CRC patients. The relative abundance of genes of the pentose phosphate pathway is critical for cancer cells due to the generation of high levels of NADPH, which may be utilized for the nucleic and fatty acids synthesis and in the cell survival under stress conditions [321]. Moreover, a significant over-representation of genes for energy metabolism such as oxidative phosphorylation, methane metabolism, and sulfur metabolism as well as for lipopolysaccharide biosynthesis were found increase in OB-CRC patients with respect to L-CRC patients and L-HC controls. Sulfur-metabolizing microbes, which convert dietary sources of sulfur into genotoxic hydrogen sulfide (H_2S), have been previously associated with development of CRC [322]. Moreover, gut-derived H_2S may fragment the mucus bilayer of the gastrointestinal tract and this breach may precede tumorigenesis by exposing gut epithelium to immunogenic luminal bacteria [323]. The significant increase of genes for lipopolysaccharide biosynthesis found in the OB-CRC groups could be in part attributed to the significant increase in the abundance of *Escherichia coli* and other species of the family Enterobacteriaceae, which contain specific enzymes to produce LPS [324]. These results suggest that the microbial differences observed in OB-CRC patients could be associated with changes in functional pathways.

In addition, we have also demonstrated the existence of a significant association between the gut microbiota and the anti-cancer response of CRC patients treated with neoadjuvant radiochemotherapy. Moreover, we have found that some microbial-derived metabolites such as SCFAs could be at least partially responsible of the response to

radiochemotherapy in these CRC patients. Finally, we have identified a consortium of baseline CRC-enriched bacteria (*Ruminococcus* albus, *Bifidobacterium bifidum*, *Faecalibacterium prausnitzii*, *Fusobacterium* nu*cleatum*, and *Bacteroides fragilis*), which may potentially serve as diagnostic bacterial markers of good or bad response to neoadjuvant radiochemotherapy.

We observed that gut microbiota composition was relatively stable over treatment time following radiochemotherapy treatment, with the exception of a significant decrease in the abundance of *Fusobacterium*, *Escherichia* and *Klebsiella* and a significant increase in *Bifidobacterium* at post-treatment time compared to baseline. *Klebsiella* and *Fusobacterium* are pathogens normally found in the human intestine that cause diarrhea, bloodstream infections and considerably increase the rates of treatment failure and death [325].

After treatment, CRC patients were classified as responders (N) versus non-responders (NR) based on their good or poor response to the radiochemotherapy. Interestingly, we found significant differences in alpha diversity at genus level, with an increase in the diversity (Shannon) and richness (Chao 1) in R compared to NR patients. Similarly, there was a statistically significant difference in beta diversity (Bray-Curtis dissimilarities and Jaccard index), finding a notable clustering effect by response status in the gut microbiome of these patients, indicating a difference in bacterial community composition between R and NR patients.

At taxa levels we found a significant enrichment of probiotic and butyrate producer-bacteria such as *Bifidobacterium bifidum*, *Ruminoccous albus*, *Roseburia* and *Faecalibacterium prausnitzii* in R patients, while NR patients showed an enrichment of unfavorable microbial taxa such as *Fusobacterium nucleatum*, *Bacteroides fragilis*,

Escherichia coli, *Prevotella copri* and *Klebsiella*. Several studies have shown that butyrate-producing bacteria are negatively related to irritable bowel disease and CRC [326, 327].

Additionally, both *Fusobacterium* and *Prevotella* have been related to recurrent CRC after chemotherapy. Given that *Fusobacterium nucleatum* has been previously correlated with chemoresistance [154], our results may suggest that the higher load of *Fusobacterium nucleatum* present in NR patients could be a potential promoter of CRC chemoresistance and therefore of a poor response to CRC treatment. Similarly, the enterotoxigenic *Bacteroides fragilis* also enriched in the NR patients is a significant source of chronic inflammation and it has been previously associated with the development and aggressiveness of CRC and poor patient outcome [280, 328]. Afterwards, these data suggest that gut microbiota composition of the R patients shifted towards a microbial profile that has great similarity to the gut microbiota of healthy control.

Next, we sought to gain insight into the mechanism through which the gut microbiome may influence response to radiochemotherapy. Regarding metabolic function of gut microbiota, in the current study Picrust analysis showed significant differences between R and NR patients. In NR patients we have found an increase in the abundance of genes for lipopolysaccharide biosynthesis as well as for araquidonic acid metabolism, and gluta-thione metabolism compared to R patients. The significant increase of genes for lipo-polysaccharide biosynthesis could be related to the significant increase in the abundance of gram-negative bacteria such as *Escherichia coli* in the NR patients, these bacteria contain specific enzymes to produce LPS, that can induce toll-like receptor 4 signaling and promote cell survival and proliferation in CRC patients [329]. Similarly, the arachidonic acid pathway is important in the development and progression of numerous

malignant diseases, including CRC, due that araquidonic acid stimulates key downstream signaling cascades that regulate cell proliferation, apoptosis, angiogenesis, inflammation, and immune surveillance [330, 331]. With respect to the increase in gene for glutathione metabolism in NR patients, some studies have described that the elevated levels of glutathione in tumor cells are able to protect such cells in bone marrow, breast, colon, larynx, and lung cancers by conferring resistance to several chemotherapeutic drugs [332, 333]. Other bacterial functions involving metabolism of cofactors and vitamins and energy production pathways such as oxidative phosphorylation was also increase in NR patients. These pathways may serve as alternative bioenergetic sources for metabolically stressed cancer cells [334].

Remarkably, a recent metagenomic analysis reported that the CRC-associated microbiome showed an association with alterations in polyamine metabolism, indicating that these metabolites could be particularly important in CRC development and progression [335]. In our study significant differences in the serum levels of several polyamines and acetyl derivatives of polyamines were found between R and NR patients at post-treatment point. Moreover, we observed that the abundance of N1, N12-DiAcSP and N8-AcSPD were positively associated to the increase abundance of *Bacteroides fragilis* and *Fusobacterium nucleatum* in NR patients.

In fact, *Bacteroides* spp. and *Fusobacterium* spp. can synthesize putrescine and spermidine *in vitro* and *in vivo* [336]. Goodwin *et al* demonstrate that purified *Bacteroides fragilis* toxin (BFT) up-regulates spermine oxidase in HT29/c1 and T84 colonic epithelial cells, producing spermine oxidase-dependent generation of ROS and induction of a marker of DNA damage such as H2A histone family member X phosphorilated (γ-H2A.x) [337]. In another study Johnson *et al* found that following antibiotic treatment, resected colorectal cancer tissues harbored disrupted bacterial biofilms and lowered N1,

N12-DiAcSP tissue concentrations compare to biofilm-negative colon cancer tissues, suggesting that gut microbes can induce an increase of host generated N1,N12-DiAcSP [338].

Moreover, the activation of the amino acid metabolic pathways by the intestinal microbiota of NR patients could contribute to the increase of polyamines, which are actively assimilated by the cells of the intestinal epithelium and induces rapid cell proliferation and favoring the tumorigenesis [339, 340].

On the other hand, CRC has been associated to alterations in the metabolism of SCFAs, which have been shown to exhibit potential anti-carcinogenic effects in cellular and animal models of colon cancer [341, 342]. Here, we have found that R patients displayed a significant increase in the fecal abundance of several SCFAs such as acetic and butyric acid after radiochemotherapy treatment. Moreover, there was a positive correlation between the fecal levels of butyrate and the abundance of the *Faecalibacterium prausnitzii* and *Ruminoccocus albus* in these patients. *Faecalibacterium praustnitzii* is considered important in health promotion, as it is able to produce butyrate from dietary fibre and possesses anti-inflammatory properties [343]. A decrease in *Faecalibacterium prausnitzii* and butyrate levels defines microbiota dysbiosis in patients suffering inflammatory bowel disease [344]. In addition, *Faecalibacterium* is able to use the acetate produced by *Bifidobacterium* (also increased in N patients) with the subsequent modulation of the intestinal mucus barrier by modification of goblet cells and mucin glycosylation [345]. Butyrate is required for colonic epithelium repair and Treg cells production, which regulate the local immune response and suppressing colonic inflammation and carcinogenesis [199]. Moreover, butyrate has been shown to be able to induce IL-18 production in intestinal epithelial cells by activating GPR109a receptor, which stimulates the mucosal tissue repair through the regulation of the production and

availability of IL-22 [197]. The absence of IL-18 has been associated with gut microbiota dysbiosis, alterations of the inflammatory response, and a dysregulation of the homeostatic and mucosal repair, resulting in increased susceptibility to carcinogenesis [198]. In addition, after radiochemotherapy treatment we have found in the NR study group a significant decrease in the fecal levels of acetic, butyric, isobutyric, and hexanoic acids compared to R patients, indicating the exhaustion of butyric acid-producing microbiota in their colon [346]. In a previous study, hexanoic acid has been shown to reduce the coloniza-tion and dysbiotic expansion of potentially pathogenic bacteria in the gut [347].

Finally, we found that plasma zonulin levels were significantly higher in the NR patients compared to R. Increased zonulin level was associated with the abundance of Prevotella copri in R patients. Zonulin is a protein synthesized in intestinal and liver cells that reversibly modulates the intestinal permeability of the intestinal epithelial barrier by modulating intercellular tight junctions [348]. As as we have indicated previously, *Prevotella* contains enzymes that are important in mucin degradation, which may disrupt the colonic mucus barrier and increase intestinal permeability, allowing the diffusion of pathogens, toxins, and antigens from the luminal environment into the mucosal tissues and circulatory system [349], resulting in immune activation and tissue inflammation which modulate cancer initiation, progression and response to anticancer treatment [281]. Then, the significant increase in *Prevotella* abundance found in our study could be in part associated with the poor or non-response to radiochemotherapy in NR patients.

CONCLUSIONS

1.   We have demonstrated that the gut microbiota in CRC patients differs in intestinal microbiota composition in comparison with healthy controls. Moreover, there is an association between inflammation, BMI, and gut microbiota in CRC patients.

2.   In CRC patients gut microbiota is characterized by a significantly lower bacterial diversity and richness, a significant increase in proinflammatory opportunistic pathogens, and a decrease in the relative abundance of beneficial or commensal butyrate-producing bacteria compared to HC subjects. Nevertheless, the presence of obesity does not induce significant changes in the alpha diversity of intestinal bacteria of these CRC patients.

3.   Compared to L-CRC patients and L-HC controls the gut microbiota of OB-CRC patients is characterized by the presence of a higher abundance Prevotella, Fusobacterium nucleatum, Enterobacteriaceae, and Escherichia coli (opportunistic pathogens), which may impair intestinal barrier function (increased circulating zonulin levels), and may contribute to inflammatory processes related to CRC by means of increasing the production of inflammatory molecules such as IL-1β and TMAO.

4.   In addition, the presence of obesity in CRC patients is also associated to changes in the functionality of the gut microbiota, with a significant over-representation of genes for energy metabolism such as oxidative phosphorylation, methane metabolism, and sulfur metabolism as well as for lipopolysaccharide biosynthesis in OB-CRC patients with respect to L-CRC patients and L-HC controls, pathways associated with inflammation, metabolism and intestinal permeability homeostasis.

5.   Neoadjuvant radiochemotherapy treatment in CRC patients did not induce significant changes in gut microbiota diversity and composition, with the exception of a

significant decrease in Fusobacterium, Escherichia and Klebsiella and a significant increase in Bifidobacterium at post-treatment time compared to baseline.

6.   After the classification of CRC patients in responder (R) and poor or non-responder (NR) to the neoadjuvant radiochemotherapy we have observed a significant increase in the diversity and richness in R patients compared to NR.

7.   Compositional changes were showed between both study patients, with a significant enriched of probiotic and butyrate producer-bacteria in R patients, accompanied by an enriched in unfavorable pro-inflammatory bacteria in NR patients.

8.   NR patients had significantly higher levels of spermine and some acetyl derivatives of polyamines as well as serum zonulin and significantly lower levels of fecal of acetic, butyric, isobutyric, and hexanoic acids than R patients.

9.   PICRUSt analysis found in N patients an over-representation of genes involved in lipopolysaccharide biosynthesis as well as in araquidonic acid and glutathione metabolism, genes from pathways associated with bacterial pathogenesis, inflammation, cell survival, proliferation and therapy response.

10.   Finally, we have identified a consortium of baseline CRC-enriched bacteria that potentially could predict cancer treatment outcome.

These finding could provide new clues for the development of diagnostic tools for CRC prevention.

ABBREVIATIONS

5-FU	5-Fluorouracil.
AC	Cholic acid.
AICR	American Institute for Cancer Research.
AKT	Serine/threonine kinase.
AOM	Azoxymethane.
AP-1	Transcripcion factor activator protein 1.
APC gene	Adenomatous polyposis coli gene.
APC/Min mice	Adenomatous polyposis coli/multiple intestinal neoplasia mice.
AUC	Area under the curve.
BAX gene	BCL2 associated X gene.
BFT	Bacteroides fragilis toxin.
BMI	Body mass index.
CCL17	C-C motif chemokine ligand 17.
CCL20	C-C motif chemokine ligand 20 or liver activation regulated chemokine (LARC) or macrophage inflammatory protein-3 (MIP3A).
CD3	Cluster of differentiation 3.
CD8	Cluster of differentiation 8.
CD68	Cluster of differentiation 68.
CDCA	Chenodeoxycholic acid.
CDK1	Cyclin-dependent kinase 1.
CDT	Cytolethal strain toxin.
CIN	Chromosomal instability.
CpG	Dinucleotide citosin-guanine.
CPMG module	Car-Purcell-Meiboom-Gill module.

CRC   Colorectal cancers.

CRP   C-reactive protein.

CXCL2   C-X-C motif chemokine ligand 2.

CXCL8   C-X-C motif chemokine ligand 8.

CXCL9   C-X-C motif chemokine ligand 9.

CXCL10   C-X-C motif chemokine ligand 10 also known as Interferon gamma-induced protein 10 (IP-10).

D2O   Deuterium oxide.

DCA   Deoxycholic acid.

DFMO   Difluoromethylornithine.

DNA   Deoxyribonucleic acid.

DPC4 gene   Deleted in pancreatic cancer 4 gene.

DSS mice   Dextran sulfate sodium - induced colitis in mice.

ELISA   Enzyme-linked immunosorbent assay.

EPCAM gene   Epithelial cellular adhesion molecule gene.

EPIC   European Prospective Research on Cancer and Nutrition.

ERK1   Extracellularly regulated kinases 1.

ERK2   Extracellularly regulated kinases 2.

ETBF   Enterotoxigenic *Bacteroides fragilis*.

FDR   False discovery rate.

FMO   Flavin monooxygenase.

FXR   Farnesoid X receptor.

GPBAR1   G-protein-coupled bile acid receptor 1 or TGR5.

GPR41   G-protein-coupled receptor 41 for propionate.

GPR43   G-protein-coupled receptor 43 for acetate.

GPR109	G-protein-coupled receptor 109 for butyrate.
GSK3β	Glycogen synthase kinase 3 beta.
H_2S	Hydrogen sulfide.
HbA1c	Glycated hemoglobin.
HCT116	HCT116 human colon cancer cell line.
HDL	High density lipoprotein.
HT-29	HT-29 human colon cancer cell line.
IFN-γ	Interferon gamma.
IGF	Insulin-like growth factors.
IL	Interleukin.
KEGG	Kyoto Encyclopedia of Genes and Genomes.
KRAS gene	Kristen rat sarcoma virus gene (KRAS Proto-Oncogene, GTPase).
LC-MS/MS	Liquid chromatography tandem mass spectrometry.
LCA	Litholic acid.
L-CRC	Group of patients with colorectal cancer and without obesity.
LDL	Low density lipoprotein.
L-HC	Non-obese healthy controls.
LPS	Lipopolysaccharide.
MAPK	Mitogen-activated protein kinase.
miR-149	Micro RNA 149.
MLH1 gene	MutL Homolog 1 gene.
MLH3 gene	MutL Homolog 3 gene.
MMR	Mismatched base repair.
MSH2 gene	MutS Homolog 2 gene.
MSH3 gene	MutS Homolog 3 gene.

MSH6 gene	MutS Homolog 6 gene.
MSI	Microsatellite instability.
MS/MS	Tandem mass spectrometry.
MyD88	Myeloid differentiation factor 88.
N1,N12-DiAcSP	N1, N12-Diacetylspermine.
N1,N8-DiAcSPD	N1, N8- Diacetylspermidine.
N1-AcPUT	N1-Acetylputrescine.
N1-AcSP	N1-Acetylspermine.
N1-AcSPD	N1-Acetylspermidine.
N8-AcSPD	N8-Acetylspermidine.
NADPH	Nicotinamide adenine dinucleotide phosphate.
NF-kB	Nuclear factor-kappa B.
NIH	National Institutes of Health.
NIH-AARP	National Institutes of Health and American Association of Retirees.
NLRP3	NOD-like receptor family pyrin domain containing 3.
NMR	Nuclear magnetic resonance.
NOESY	Nuclear overhauser effect spectroscopy.
NR	Patients with poor or non-response to treatment.
OB-CRC	Group of patients with colorectal cancer and obesity.
ODC	Ornithine decarboxylase.
OUT	Operational taxonomic units.
PCoA	Principal coordinate analysis.
PCR	Polymerase chain reaction.
PI3K	Phosphatidyl inositol 3 kinase.

PICRUS	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States.
PIK3CA gene	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic subunit alpha gene.
PMS1 gene	Postmeiotic Segregation Increased 1 gene.
PMS2 gene	Postmeiotic Segregation Increased 2 gene.
PMS2 gene	Postmeiotic Segregation Increased 2 gene.
PUFA	Polyunsaturated fatty acid.
PUFA ω-3	Omega 3 polyunsaturated fatty acid.
PUT	Putrescine.
R	Patients with good response to treatment or responders.
RCT	Radiochemotherapy.
RF	Random Forest.
ROS	Reactive oxygen species.
SCFA	Short chain fatty acids.
SMAD4 gene	SMAD family member 4 gene.
SMO	Spermine oxidase.
SP	Spermine.
SPD	Spermidine.
STAT3	Signal transducer and activator of transcription 3.
T84	Transplantable human carcinoma cell line derived from a lung metastasis of a colon carcinoma (colonic epithelial cells).
TCA	Citrate cycle.
TGF/BRII	Transforming growth factor beta receptor 2.
TGR5	G-protein-coupled receptor 5 or GPBAR1.

TIGIT	T-cell immune receptor with immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domains.
TLR	Toll-like receptors.
TMA	Trimethylamine.
TMAO	Trimethylamine N-oxide.
TNF	Tumor necrosis factor.
TNF-α	Tumor necrosis factor alpha.
TP53 gene	Tumor protein 53 gene.
TSP	TopSpin program.
UHPLC	Ultra-high performance liquid chromatography.
WCRF	World Cancer Research Fund.
WHO	World Health Organization.
Wnt	Wingless-related integration site.
γ-H2A.x	H2A histone family member X phosphorylated.

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RESUMEN (ESPAÑOL)

PAPEL DE LA COMPOSICIÓN DE LA MICROBIOTA INTESTINAL EN EL CÁNCER COLORRECTAL Y EN LA RESPUESTA A LA RADIOQUIMIOTERAPIA NEOADYUVANTE PREVIA A CIRUGÍA

Vivimos en una era en la que el acceso a la atención médica, el diagnóstico y el tratamiento de las enfermedades ha mejorado, lo que ha tenido un impacto en la esperanza de vida media en la mayoría de las regiones del mundo. Como resultado de estas mejoras médicas, la mortalidad asociada a enfermedades ha disminuido de manera general, sin embargo, la mortalidad asociada a la patología del cáncer ha aumentado en las últimas décadas. Según los últimos datos analizados por la Organización Mundial de la Salud (OMS), el cáncer es la segunda causa de muerte en el mundo, se estima que en 2018 esta enfermedad provocó 9,6 millones de muerte, siendo responsable de una de cada seis muertes en el mundo.

El cáncer colorrectal (CRC, del inglés "colorectal cancer") es en la actualidad el segundo tipo de cáncer que mayor número de muertes causa al año a nivel mundial, con una mayor incidencia en los países industrializados, ya que el riesgo de desarrollar CRC, además de estar relacionado con la heredabilidad genética y con la edad, se asocia con una gran variedad de factores ambientales que incluyen factores culturales y sociales. El estilo de vida de los países occidentales ha contribuido sustancialmente al aumento de la tasa de CRC en las dos últimas décadas. Los factores que influyen en el estilo de vida incluyen el consumo de alcohol, el tabaquismo, el estilo de vida sedentaria, la dieta y la obesidad.

Así, diversos estudios, han demostrado que la microbiota intestinal es otro factor ambiental crítico que contribuye al desarrollo y a la progresión del CRC, potencialmente a través de una respuesta inflamatoria, de los niveles de algunos metabolitos producidos por las bacterias y de la interferencia con el equilibrio energético de las células

cancerosas. Además, el CRC se ha asociado con una disbiosis en la composición microbiana asociada al tumor y a la mucosa adyacente.

La obesidad está reconocida como un factor que influye sobre diferentes enfermedades, entre las que se encuentran varios tipos de cánceres y enfermedades crónicas. La obesidad está considerada un factor de riesgo muy importante en el CRC, hasta tal punto que el 14% de los casos de CRC se atribuyen al sobrepeso o a la obesidad. La existencia de una relación entre la obesidad y el CRC ha sido ampliamente observada, tanto en estudios epidemiológicos como en estudios con modelos animales. Aunque los mecanismos subyacentes a esta relación no han sido desenmarañados completamente, son los procesos inflamatorios los que han ganado atención con respecto a esta asociación. La obesidad existe vinculada a un estado inflamatorio de bajo grado y el proceso de carcinogénesis se ve favorecido por la inflamación, es por ello que la obesidad aumenta el riesgo de padecer CRC. Si se indaga más en el proceso inflamatorio, ya sea inducido o potenciado por la obesidad, encontramos que la inflamación está causada en gran parte por un aumento en la infiltración de los macrófagos en el tejido adiposo, lo que induce la secreción de citocinas inflamatorias, como IL-6, TNF-α y MCP-1. Estas citocinas también han sido relacionadas con el desarrollo del CRC. Hay estudios que nos ayudan a comprender estas relaciones, como el caso de un estudio reciente realizado con ratones a los que se les implantaron tumores MC38 subcutáneamente, donde la obesidad inducida por una dieta alta en grasa fue asociada con un mayor desarrollo de tumores, además del aumento de la adiposidad local, una mayor concentración de macrófagos en el tejido y un aumento de la inflamación. La obesidad influye sobre el organismo a distintos niveles, en el intestino es capaz de inducir alteraciones en la barrera de la mucosa intestinal, esta modificación provoca un aumento de la permeabilidad intestinal y como consecuencia se produce un incremento de la endotoxemia metabólica, la cual actúa de manera conjunta con la

inflamación existente en el tejido adiposo potenciando la producción de citocinas inflamatorias, de esta forma la inflamación resultante podría favorecer la carcinogénesis.

A nivel intestinal la inflamación está muy unida a la composición de la microbiota intestinal, la cual también ha sido vinculada con la obesidad y con el CRC. La microbiota intestinal puede inducir la inflamación, la alteración en la permeabilidad intestinal, la toxicidad bacteriana y puede potenciar al sistema inmunológico, siendo todo esto causas posibles del desarrollo de la carcinogénesis. Además, estos efectos causados por la microbiota intestinal se ven potenciados en caso de disbiosis, que está asociada con la obesidad. Por tanto, a la vista de las relaciones conocidas, obesidad con CRC, obesidad con microbiota intestinal y CRC con microbiota intestinal, se nos plantea la existencia de una red de interacciones que asocia a los tres factores: CRC, obesidad y microbiota intestinal. Esta triple relación ha sido asumida como un mayor riesgo de padecer cáncer provocado por los cambios fisiológicos derivados de la obesidad asociada a una disbiosis intestinal.

El N-óxido de trimetilamina (TMAO) es un metabolito derivado del metabolismo de las bacterias, su origen se encuentra en la acción de la microbiota intestinal sobre la colina y la L-carnitina de la dieta, que están contenidos en las carnes rojas y otros alimentos de origen animal. Los niveles plasmáticos de TMAO han sido asociados con un mayor riesgo de padecer CRC, obesidad, enfermedades cardiovasculares y diabetes. Los niveles plasmáticos de TMAO parecen estar relacionados tanto con el riesgo de desarrollar CRC como con un pronóstico desfavorable de su evolución. Se sabe que TMAO participa en la inducción de citocinas inflamatorias, como IL-6 y TNF-α, lo que propone a la inflamación como vínculo entre TMAO y el CRC. Y, por tanto, TMAO podría ser un indicador intermediario de la relación entre la microbiota intestinal y el CRC.

Por otra parte, hoy en día la radioquimioterapia neoadyuvante seguida de cirugía se ha convertido en un procedimiento estándar en el tratamiento de pacientes con CRC. Son varios los estudios que han sugerido que la disbiosis intestinal puede influir en la respuesta del cuerpo a una variedad de terapias contra el cáncer, que incluyen radioterapia, quimioterapia e inmunoterapia. Estudios recientes han señalado a la microbiota intestinal como un factor capaz de influir en la respuesta farmacológica y en la toxicidad de los pacientes con CRC sometidos a tratamiento a través de mecanismos clave, como la translocación, la inmunomodulación, el metabolismo, la reducción de la diversidad y la variación ecológica. Hasta el momento, se ha demostrado que aproximadamente cuarenta fármacos quimioterapéuticos son metabolizados por la microbiota intestinal. Además, se han identificado bacterias intestinales específicas capaces de modificar la efectividad de los tratamientos contra el cáncer a través del metabolismo directo de los fármacos y/o la modulación de la respuesta inmune del hospedador, como especies de *Bifidobacterium*, *Lactobacillus* y *Streptococcus* que alivian la diarrea y la toxicidad inducida por la quimioterapia basada en irinotecán en pacientes con CRC, *Lactobacillus rhamnosus* que tiene un efecto sinérgico con la aplicación de radioterapia en las actividades antiproliferativas y antiinflamatorias o *Veillonella dispar* y el género *Sutterella* asociados a la quimiorresistencia. Y se sabe que varios filos bacterianos median en el metabolismo de los fármacos a través de reacciones como degradación proteolítica, la escisión de isoxazol, la desnitrificación, la desconjugación, la acetilación/desacetilación, la formación y/o hidrólisis de aminas, así como por adherencia física a los fármacos. Scott y cols. describieron la influencia de la microbiota intestinal en la eficacia de las fluoropirimidinas (uno de los tratamientos de primera línea para el CRC) a través de una interconversión del fármaco que involucra la vitamina B6 y B9 bacteriana y el metabolismo de los ribonucleótidos. También se ha visto

que el efecto letal del 5-fluorouracilo sobre las células de CRC puede ser incrementado bajo la influencia de los metabolitos de la microbiota intestinal. En concreto se ha señalado el papel de *Fusobacterium nucleatum,* el cual es capaz de promover la resistencia del CRC frente a la quimioterapia mediante vías de señalización de la inmunidad innata a través de TLR-4, MYD88 y de microARNs específicos capaces de activar la vía de la autofagia, alterando la respuesta quimioterapéutica en el CRC. Además de la acción de las bacterias sobre los fármacos, la radiación provoca alteraciones en la composición de la microbiota intestinal. Recientemente se ha evaluado la composición de la microbiota fecal de ratones que habían sido sometidos a radiación y se vio que la radiación inducía alteraciones significativas en la composición microbiana del tracto intestinal a nivel de género. Por lo tanto, aunque se conoce la influencia de la microbiota intestinal en la regulación de la respuesta del hospedador a la radioterapia, los mecanismos implicados no están totalmente determinados. No obstante, ha sido sugerido que si la radioterapia ejerce respuestas antitumorales que están mediadas por la respuesta inmune, la microbiota intestinal podría jugar un papel importante en el efecto inmunogénico de dicha radioterapia.

Por otro lado, las bacterias producen metabolitos microbianos que pueden modificar la proliferación de las células cancerosas y la respuesta a la quimioterapia, como se ha visto en un estudio de cáncer de mama. Ross y cols. vincularon al butirato y al propionato (dos ácidos grasos de cadena corta derivados del metabolismo bacteriano) con una respuesta patológica completa a la quimioterapia neoadyuvante en mujeres con cáncer de mama en estadio temprano. De estos ácidos grasos de cadena corta se sabe que pueden inducir disfunción en la barrera epitelial intestinal, activando mediadores inflamatorios como las citocinas IL-6 y TNF-α que dañan las células epiteliales y sus uniones. Otro tipo de metabolito derivado de las bacterias son las poliaminas, entre ellas la espermina, la

espermidina y la putrescina, que han sido implicadas en casi todos los pasos de la carcinogénesis colorrectal. Las poliaminas son moléculas indispensables en el crecimiento celular normal, en la expresión génica y son necesarias en la proliferación celular, pero sus concentraciones van en aumento durante la transición de una célula sana a una célula tumoral. Recientemente se ha demostrado que las poliaminas acetiladas son más específicas para el cáncer. Por ejemplo, se ha visto que N1, N12-diacetilespermina sufre un considerable aumento en los casos de CRC y también en las lesiones colorrectales displásicas.

En la presente tesis doctoral hemos tratado de descifrar las incógnitas presentes en la asociación entre la composición de la microbiota intestinal y el CRC incluyendo la respuesta a la radioquimioterapia neoadyuvante previa a cirugía. En primer lugar, nos fijamos objetivos para determinar la composición y las funciones de la microbiota intestinal en muestras fecales de pacientes con CRC con (OB-CRC) y sin obesidad (L-CRC), para ello comparamos los perfiles de microbiota intestinal presentes en pacientes OB-CRC y L-CRC con la microbiota de controles sanos no obesos (L-HC). Además, se analizarón los niveles fecales de TMAO, la permeabilidad intestinal y el estado inflamatorio en los diferentes grupos de estudio para así tratar de desvelar la posible relación entre la microbiota intestinal los niveles plasmáticos de TMAO, el estado inflamatorio y la permeabilidad intestinal en el contexto del CRC asociado a la obesidad.

Para llevar a cabo el estudio se reclutaron 45 pacientes con CRC en estadíos II-III (T2-T4 y/o N1-N2) cuyo rango de edad fue 35-75 años y que fueron clasificados según su IMC (de acuerdo a los criterios de la OMS) en no obesos (IMC < 30 kg/m^2) (L-CRC) y obesos (IMC ≥ 30 kg/m^2) (OB-CRC), además de un grupo control formado por 20 sujetos sanos y no obesos (L-HC). A estos pacientes y controles se les recogieron muestras de sangre y de heces a nivel basal, previo a cualquier tipo de tratamiento. En dichas muestras

de sangre se realizaron análisis de los niveles de glucosa sérica, colesterol total, triglicéridos, colesterol HDL, colesterol LDL y hemoglobina glucosilada (HbA1c) por métodos enzimáticos. Además, en muestras de plasma se midieron los niveles de zonulina mediante ELISA y los de TMAO fueron cuantificados mediante resonancia magnética nuclear en muestras de suero. Las muestras de heces fueron utilizadas para el análisis de ARNr 16S mediante secuenciación NGS utilizando plataforma Ion S5 (ThermoFisher) y los datos obtenidos tras la secuenciación fueron analizados mediante la plataforma Qiime2 y PICRUSt (Galaxy).

Cuando comparamos la composición de la microbiota fecal de los tres grupos de estudio (OB-CRC, L-CRC y L-HC) se observó que los dos grupos de pacientes con CRC tenían mayor similitud entre sí que con el grupo control. El análisis de la diversidad alfa (composición de la comunidad) del microbioma intestinal de los tres grupos reveló una disminución de la riqueza (índice Chao1) y la diversidad (índice de Shannon) en los grupos OB-CRC y L-CRC en comparación con los controles L-HC. Sin embargo, no se encontraron diferencias significativas en los índices de Chao1 y Shannon entre los grupos OB-CRC y L-CRC. Estos resultados pueden sugerir que la disminución en la diversidad de la microbiota intestinal de los pacientes con CRC no podría estar completamente relacionada con la presencia de obesidad. Además, el análisis de disimilitud de Bray-Curtis para detectar diferencias de la comunidad microbiana en la estructura mostró una agrupación de los pacientes con OBC-CRC y L-CRC juntos, pero separados de los controles L-HC, lo que sugiere que la obesidad no introduce cambios importantes en la estructura general de la comunidad microbiana intestinal de pacientes con CRC. No obstante, el presente estudio demostró que los pacientes con CRC exhiben claras diferencias en la composición de la microbiota intestinal en comparación con los individuos sanos, independientemente del IMC del paciente. Se encontraron diferencias

significativas entre los grupos a nivel de filo para Bacteroidetes, Firmicutes, Fusobacteria y Proteobacteria. De las 20 familias bacterinas presentes en los tres grupos de estudio, se encontraron diferencias significativas en la abundancia de Bacteriodaceae, Porphyromonaceae, Prevotellaceae, Streptococaceae, Clostridiaceae, Ruminococaceae, Fusobacteriaceae, Desulfovibriaceae, Enterobacteriaceae y Victivallaceae. Los análisis a nivel de género confirmaron que la microbiota intestinal de los pacientes con CRC con o sin obesidad se caracteriza por una reducción de las bacterias productoras de butirato (*Butyricimonas*, *Roseburia*, *Blautia*, *Faecalibacterium* y *Ruminococcus*) y un aumento de especies bacterianas nocivas que podrían actuar como patógenos oportunistas con propiedades proinflamatorias y procarcinogénicas (*Fusobacterium*, *Clostridium*, *Prevotella*, *Desulfovibrio* y *Enterococcus*) en comparación con los controles L-HC. Finalmente, se observó un aumento significativo en la abundancia de los taxones de patógenos oportunistas en el microbioma intestinal de pacientes OB-CRC en comparación con sujetos L-CRC y L-HC. El perfil microbiano encontrado en los pacientes OB-CRC, podría ser responsable de los niveles séricos significativamente más altos de zonulina (marcador de permeabilidad intestinal), TMAO (metabolito microbiano relacionado con la ECV) e IL-1β (factor proinflamatorio) y los niveles más bajos de IL-10 (factor antiinflamatorio) en comparación con los controles y pacientes con CRC no obesos. Estudios previos han descrito el aumento de los niveles circulantes de TMAO en pacientes con sobrepeso u obesidad y también en pacientes con CRC, en el presente estudio encontramos que la presencia de ciertos taxones bacterianos específicos en las heces humanas de ambos grupos con CRC se asociaron con la concentración de TMAO en plasma. Observamos que las concentraciones séricas de TMAO se asociaron significativa y positivamente con la abundancia de la familia Enterobacteriaceae y la especie *Escherichia coli* en pacientes OB-CRC y la abundancia de *Desulfovibrio* en

pacientes L-CRC. De acuerdo con nuestros resultados, otros estudios en humanos y animales han sugerido que varias familias de bacterias están involucradas en la producción de TMA/TMAO como Prevotellaceae y Enterobacteriaceae. Por lo tanto, el aumento de bacterias patógenas específicas como *Escherichia coli* en pacientes OB-CRC puede ser responsable (al menos parcialmente) del aumento significativo de moléculas proinflamatorias derivadas del metabolismo bacteriano como TMAO. Estudios recientes han sugerido que el TMAO podría desempeñar un papel clave en el proceso inflamatorio y que esta inducción de la inflamación podría ser un posible factor que proporcione un vínculo entre el TMAO y el cáncer. No obstante, los niveles de TMAO en sangre no sólo dependen de la composición de la microbiota intestinal y de su actividad metabólica, sino también del funcionamiento de la barrera intestinal que controla el acceso de las moléculas entre el intestino y el torrente sanguíneo. En consecuencia, en nuestro estudio encontramos que los niveles plasmáticos de zonulina fueron significativamente más altos en el grupo OB-CRC en comparación con los grupos L-CRC y L-HC. Además, este aumento del nivel de zonulina se asoció con la abundancia de *Prevotella* en pacientes con OB-CRC. Una bacteria asociada a la degradación de mucina de la capa de mucosa de la barrera intestinal y en consecuencia al aumento de la permeabilidad intestinal.

Con respecto a la microbiota específica, significativamente incrementada en pacientes OB-CRC, otros autores han descrito un incremento de *Fusobacterium nucleatum* en personas obesas que en comparación con personas delgadas. Es más, nosotros hemos descrito una asociación de esta especie bacteriana con la abundancia de IL-1β en pacientes OB-CRC, una interleucina proinflamatoria que induce la activación de la vía Wnt, una vía clave para la tumorogénesis intestinal. Estos datos podrían respaldar el papel de esta molécula en la patogenia del CRC. Por otro lado, la presencia de niveles bajos IL-10 en pacientes OB-CRC se asociaron con una disminución en la abundancia de *Blautia* y

Faecalibacterium prausnitzii, ambas bacterias son importantes proveedores de butirato para el epitelio colónico. El butirato es un ácido graso de cadena corta que contribuye a mantener la integridad de la barrera intestinal y disminuir la permeabilidad intestinal.

Finalmente, los análisis de funcionalidad de la microbiota intestinal encontrada en los tres grupos de estudio, sugieren una menor abundancia relativa de genes responsables de las funciones del metabolismo de los carbohidratos, como el metabolismo del butanoato y la vía de la pentosa fosfato, junto con genes responsables del metabolismo de aminoácidos y el procesamiento de proteínas en el retículo endoplásmico en pacientes con OB-CRC en comparación con pacientes con L-CRC. La abundancia de genes de la vía de la pentosa fosfato es fundamental para las células cancerosas debido a la generación de niveles elevados de NADPH, que pueden utilizarse para la síntesis de ácidos grasos y nucleicos y para la supervivencia celular en condiciones de estrés. Además, en estos pacientes OB-CRC encontramos una sobrexpresión de genes implicados en el metabolismo energético, como la fosforilación oxidativa, el metabolismo del metano y el metabolismo del azufre, así como para la biosíntesis de lipopolisacáridos con respecto a los pacientes L-CRC y los controles L-HC. Los microbios metabolizadores del azufre, que convierten las fuentes dietéticas de azufre en sulfuro de hidrógeno genotóxico (H_2S), se han asociado previamente con el desarrollo de CRC. Además, el H_2S derivado del intestino puede fragmentar la bicapa mucosa del tracto gastrointestinal y esta ruptura puede preceder a la tumorigénesis al exponer el epitelio intestinal a bacterias luminales inmunogénicas. Finalmente, el aumento significativo de genes para la biosíntesis de lipopolisacáridos encontrados en el grupo OB-CRC podría atribuirse en parte al aumento significativo de la abundancia de *Escherichia coli* y otras especies de la familia Enterobacteriaceae, que contienen enzimas específicas para producir LPS.

En conclusión, en este estudio se ha puesto de manifiesto la existencia de una asociación entre la inflamación, el IMC y la microbiota intestinal de los pacientes con CRC. En primer lugar, mostramos que la obesidad no induce cambios significativos ni en la diversidad ni en la riqueza de las bacterias intestinales de los pacientes con CRC. En segundo lugar, demostramos que la presencia de obesidad en pacientes con CRC está asociada a cambios en la composición y en la funcionalidad de la microbiota intestinal. Así, la microbiota intestinal de los pacientes con CRC y con obesidad se caracteriza por la presencia de una mayor abundancia de patógenos oportunistas (como *Prevotella*, *Fusobacterium nucleatum*, *Enterobacteriaceae* y *Escherichia coli*), que pueden alterar la función de la barrera intestinal (determinado por el aumento de los niveles de zonulina circulante), y pueden contribuir a los procesos inflamatorios relacionados con el CRC mediante el aumento de la producción de moléculas inflamatorias como IL-1β y TMAO. Aunque es posible que nuestro estudio tenga algunas limitaciones en el análisis estadístico debido a las múltiples pruebas y debería replicarse en cohortes más grandes (incluidas otras poblaciones con diferentes patrones de alimentación y hábitos culturales), en general, nuestros resultados sugieren un papel importante la microbiota intestinal en el desarrollo de CRC en pacientes con obesidad. Además, estos hallazgos podrían proporcionar nuevas pistas para el desarrollo de herramientas de diagnóstico para la prevención del CRC.

Por otra parte en la presente tesis doctoral hemos establecido las diferencias en la composición y en la diversidad de la microbiota intestinal entre pacientes con CRC y controles sanos; estudiado los cambios en la diversidad y composición de la microbiota intestinal de los pacientes con CRC antes, durante y después de completar el tratamiento con radioquimioterapia neoadyuvante previo a cirugía y analizado las diferencias en la composición y función de la microbiota intestinal después de la clasificación de los

pacientes con CRC en buenos (respondedores) y malos o no respondedores (no respondedores) a la radioquimioterapia neoadyuvante. Además, hemos medido metabolitos producidos por la microbiota intestinal (como los ácidos grasos de cadena corta y las poliaminas), marcadores de inflamación y de permeabilidad intestinal antes y después de la radioquimioterapia en pacientes respondedores y no respondedores y estudiado su relación con la composición de su microbiota intestinal. Finalmente, hemos establecido si la composición inicial de la microbiota podría predecir la respuesta al tratamiento con radioquimioterapia neoadyuvante en pacientes con CRC.

Para llevar a cabo esta parte del estudio se reclutaron 40 pacientes con CRC en estadíos II-II (T2-T4 y/o N1-N2) y con rango de edad entre 35-75 años, que recibieron tratamiento con radioquimioterapia neoadyuvante durante 5 semanas (radioterapia pélvica (50 Gy en fracciones de 2 Gy/sesion) y Capecitabina oral (825 mg/m^2/12h). También se incluyó un grupo control de 20 sujetos sanos pareados por edad, sexo e IMC con el grupo de pacientes. En el grupo de pacientes, las muestras de sangre y heces se recogieron al inicio (T0), 2 y 4 semanas después del inicio de la radioquimioterapia (T1 y T2) y al final del tratamiento (7 semanas después de finalizar el tratamiento) (T3). Las muestras se combinaron en tres grupos: basal (T0), puntos de tratamiento (T1 y T2) y postratamiento (T3).

Las muestras de heces fueron utilizadas para el análisis de ARNr 16S mediante secuenciación NGS utilizando plataforma Ion S5 (ThermoFisher) y los datos obtenidos tras la secuenciación fueron analizados mediante la plataforma Qiime2 y Picrust (Galaxy). Los niveles de poliaminas séricas se determinaron mediante cromatografía líquida junto con una espectrometría de masas de alto rendimiento y la extracción y el análisis de los ácidos grasos de cadena corta se realizaron en muestras de heces mediante

cromatografía de gases con detector de ionización de llama (GC-FID). Finalmente, en muestras de plasma se midieron los niveles de zonulina mediante ELISA.

En este estudio, al comparar la microbiota intestinal presente en las muestras de heces de pacientes con CRC y controles sanos encontramos una disminución significativa en la diversidad (índice de Shannon) y riqueza (índice de Chao1) en las muestras de los pacientes con CRC. Además, el análisis de beta-diversida de Bray-Curtis mostró que los pacientes con CRC se agruparon en un cluster diferente a los controles sanos, lo que sugiere importantes diferencias en la estructura de la microbiota intestinal entre pacientes con CRC y controles sanos.

En relación con la composición de la microbiota intestinal, el análisis de los perfiles de la microbiota intestinal entre ambos grupos de estudio mostró diferencias significativas en la abundancia de diferentes taxones. A nivel de filo, en comparación con los controles sanos, encontramos un incremento significativo en la abundancia relativa de Fusobacteria, Firmicutes y Lentisphaerae y Proteobacterias en los pacientes con CRC, acompañada por un descenso en la abundancia de Bacteroidetes y Actinobacteria. La microbiota intestinal de los pacientes con CRC se estuvo enriquecida en patógenos oportunistas proinflamatorios y disminuida en bacterias productoras de butirato, esenciales para el mantenimiento de la homeostasis intestinal.

Por otra parte, al analizar los posibles cambios en la diversidad y composición de la microbiota intestinal de los pacientes con CRC en respuesta al tratamiento con radioquimioterapia neoadyuvante en pacientes con CRC, se observó que la composición de la microbiota intestinal era relativamente estable durante el tiempo de tratamiento. Encontramos que los principales filos bacterianos, Firmicutes y Bacteroidetes se mantuvieron estables con el tiempo, mientras que *Fusobacterium* y Proteobacterias

disminuyeron significativamente en T3 en comparación con T0 en pacientes con CRC. Además, aunque las proporciones de las familias y géneros bacterianos difirieron entre los diferentes tiempos del seguimiento, no mostraron alteraciones significativas durante el tratamiento con radioquimioterapia excepto para los géneros *Fusobacterium*, *Escherichia* y *Klebsiella*, que disminuyeron significativamente, y el género *Bifidobacterium* que aumentó significativamente en T3 en comparación con T0.

Una vez finalizado el tratamiento (T3), los pacientes con CRC se clasificaron como respondedores (N) versus no respondedores (NR) en función de su buena o mala respuesta a la radioquimioterapia. Despues de dicha clasificación, encontramos diferencias significativas en la diversidad alfa a nivel de género, con un aumento en la diversidad (Shannnon) y la riqueza (Chao 1) en R en comparación con los pacientes NR. De manera similar, hubo una diferencia estadísticamente significativa en la beta diversidad (disimilitud de Bray-Curtis e índice de Jaccard), encontramos un efecto de agrupamiento notable en función del estado de respuesta del microbioma intestinal de estos pacientes, lo que indica una diferencia en la composición de la comunidad bacteriana entre R y NR pacientes.

Al estudiar los cambios composicionales en la microbiota intestinal a nivel taxonomico, encontramos un enriquecimiento significativo en pacientes R de bacterias productoras de probióticos y butiratos como *Bifidobacterium*, *Ruminoccous*, *Roseburia* y *Faecalibacterium praustnizii*, mientras que los pacientes NR mostraron un enriquecimiento en taxones microbianos desfavorables como *Fusobacterium nucleatum*, *Bacteroides fragilis*, *Escherichia coli*, *Prevotella copri* y *Klebsiella*. Al mismo tiempo, tanto *Fusobacterium* como *Prevotella* se han relacionado con CRC recurrente después de la quimioterapia, especialmente *Fusobacterium nucleatum* se ha correlacionado con quimiorresistencia, lo que sugiere que la mayor abundancia de *Fusobacterium nucleatum*

presente en pacientes NR es un promotor potencial de la quimiorresistencia y, por lo tanto, de una mala respuesta al tratamiento en estos pacientes. De manera similar, *Bacteroides fragilis* enterotoxigénico también enriquecido en los pacientes NR es una fuente significativa de inflamación crónica y se ha asociado previamente con el desarrollo y la agresividad del CRC y la mala evolución del paciente. Estos datos sugieren que la composición de la microbiota intestinal de los pacientes R cambió hacia un perfil microbiano que tiene una gran similitud con la microbiota intestinal de controles sanos

A continuación, buscamos conocer el mecanismo a través del cual el microbioma intestinal puede influir en la respuesta a la radioquimioterapia. Con respecto a la función metabólica de la microbiota intestinal, en el estudio actual, el análisis de funcionalidad bacteriana mostró diferencias significativas entre los pacientes R y NR. En los pacientes NR hemos encontrado un aumento en la abundancia de genes implicados en la biosíntesis de lipopolisacáridos, así como para el metabolismo del ácido araquidónico y el metabolismo del glutatión en comparación con los pacientes R. El aumento significativo de genes para la biosíntesis de lipopolisacáridos podría estar relacionado con el aumento significativo en la abundancia de bacterias gram-negativas como *Escherichia coli* en los pacientes NR, esta bacteria contiene enzimas específicas para producir LPS, que pueden promover la supervivencia y la proliferación celular en pacientes con CRC. Otras funciones bacterianas que involucran el metabolismo de cofactores y vitaminas y vías de producción de energía, como la fosforilación oxidativa, también estuvieron aumentadas en pacientes NR. Estas vías pueden servir como fuentes bioenergéticas alternativas para las células cancerosas sometidas a estrés metabólico.

Un análisis metagenómico reciente ha descrito la asociación de la microbiota intestinal de pacientes con CRC con alteraciones en el metabolismo de las poliaminas. En nuestro estudio hemos encontrado diferencias significativas en los niveles séricos de varias

poliaminas y derivados acetilados de poliaminas entre pacientes R y NR. Además, observamos que la abundancia de N1, N12-DiAcSP y N8-AcSPD se asociaron positivamente con el aumento de la abundancia de *Bacteroides fragilis* y *Fusobacterium nucletum* en pacientes NR.

Con respecto al metabolismo de los AGCC, hemos encontrado en pacientes R un aumento significativo en la abundancia fecal de varios AGCC como el ácido acético y el butírico. Además, hubo una correlación positiva entre los niveles fecales de butirato y la abundancia de *Faecalibacterium praustnizii* y *Ruminoccocus albus* en estos pacientes.

Finalmente, encontramos que los niveles plasmáticos de zonulina eran significativamente más altos en los pacientes NR en comparación con R. El aumento del nivel de zonulina se asoció con la abundancia de *Prevotella copri* en los pacientes R.

En conclusión, este estudio mostró que la microbiota intestinal en pacientes con CRC difiere en la composición de la microbiota intestinal en comparación con los controles sanos. El CRC se asoció con una diversidad y riqueza de microbiota significativamente menor, una abundancia relativa significativamente mayor de patógenos oportunistas proinflamatorios y una abundancia menor de bacterias beneficiosas o comensales productoras de butirato.

Además, no hemos encontrado cambios significativos en la diversidad y composición de la microbiota intestinal en respuesta a la radioquimioterapia neoadyuvante en pacientes con CRC, con la excepción de una disminución significativa de *Fusobacterium*, *Escherichia* y *Klebsiella* y un aumento significativo de *Bifidobacterium* en el tiempo postratamiento en comparación con el tiempo basal. Sin embargo, después de la clasificación de los pacientes con CRC en R y NR a la radioquimioterapia neoadyuvante, hemos observado un aumento significativo en la diversidad y riqueza en pacientes R en

comparación con NR. Asimismo, se evidenció un cambio de composición entre grupos de estudio, con un importante enriquecimiento de bacterias productoras de probióticos y butirato (como *Bifidobacterium*, *Ruminoccous*, *Roseburia* y *Faecalibacterium praustnizii*) en pacientes R, acompañado de un enriquecido en bacterias proinflamatorias desfavorables (como *Fusobacterium nucleatum*, *Bacteroides fragilis*, *Escherichia coli*, *Prevotella copri* y *Klebsiella*) en pacientes NR. Además, los pacientes NR mostraron niveles significativamente más altos de algunos derivados acetilados de poliaminas y de zonulina sérica y niveles significativamente más bajos de ácido butírico fecal que los pacientes R. Estos metabolitos derivados de bacterias son factores importantes que conectan la microbiota intestinal con el CRC y podrían ser responsables de la eficacia de la radioquimioterapia. El análisis PICRUSt encontró en pacientes NR una sobrerrepresentación de genes involucrados en la biosíntesis de lipopolisacáridos, así como en el metabolismo del ácido araquidónico y glutatión, de genes de vías asociadas con la patogénesis bacteriana, la inflamación, la supervivencia celular, la proliferación y la respuesta a la terapia.

Finalmente, hemos identificado a tiempo basal T0 un consorcio de bacterias enriquecidas con CRC (*Ruminococcus albus*, *Bifidobacterium bifidum*, *Faecalibacterium praustnizii*, *Fusobacterium nucleatum*) que potencialmente podrían predecir la respuesta a la radioquimioterapia adyuvante. Indicando, que la composición de la microbiota inicial es decir a T0 de pacientes con CRC es importante para predecir la respuesta a la radioquimioterapia neoadyuvante. Entonces, una microbiota intestinal sana es indispensable para una respuesta terapéutica óptima y la microbiota disbiótica podría ser la razón subyacente de la respuesta variable a estrategias terapéuticas similares en diferentes pacientes.