ROLE OF THE GUT MICROBIOTA, DIET, AND OBESITY IN COLORECTAL CANCER RISK

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This dissertation is dedicated to my nephew Theo, the cutest of all who always makes me smile

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ACF	aberrant crypt foci
AOM	azoxymethane
APC	adenomatous polyposis coli
ASV	amplicon sequence variant
BMI	body mass index
CIMP	CpG island methylator phenotype
CIN	chromosomal instability
CRC	colorectal cancer
FAP	familial adenomatous polyposis
FMT	fecal microbial transplant
HFD	high fat diet
HIF	hypoxia inducible factor
HMP	Human Microbiome Project
HNPCC	hereditary nonpolyposis colorectal cancer
IP	intraperitoneal
KEGG	Kyoto Encyclopedia of Genes and Genomes
LFD	low fat diet
metaHIT	Metagenomics of the Human Intestinal Tract consortium
MSI	microsatellite instability
NCI	National Cancer Institute
NODS	nucleotide-binding oligomerization domain molecules
PF-HFD	pair-fed high fat diet, lean-HFD
PF-WD	pair-fed western diet, lean-WD
PPARγ	peroxisome proliferator-activated receptor gamma
RDP	Ribosomal Database Project
SCFA	short chain fatty acid
SEER	Surveillance, Epidemiology, and End Results
TLR	toll-like receptor
WD	western diet

ABSTRACT

In the United States, colorectal cancer (CRC) is the third most common cancer and the third leading cause of cancer mortality in men and women. Recent epidemiological evidence has shown that there's been a steady increase in young onset CRC, underlying a continued need to understand mechanisms that may be contributing to its development. One risk factor that continues to persist and rise is obesity. Obesity is a multifaceted disease characterized by various metabolic and physiologic changes that influence tumorigenesis. Another component that is altered in obesity and has been shown to contribute to CRC is the gut microbiota. Obesity associated gut microbiota is different relative to a lean counterpart and has been linked poor colonic health, which can increase risk for CRC. Researchers have shown that intestinal tumorigenesis is worse in diet induced obesity but given other related conditions like chronic inflammation, the role of the gut microbiota in obesity associated CRC risk has not been adequately isolated. To address this gap and to further explore the role of diet in this relationship given its importance in driving obesity and impacting gut microbiota composition, we performed two studies. First, we assessed the role of obesity and/or two different obesogenic diets on gut microbiota composition and intestinal permeability. We hypothesized that diet and obesity would affect gut microbial community composition and that obese mice would have higher intestinal permeability relative to lean mice regardless of diet. Our results indicated that both diet and obesity were significant predictors and had varying effects on species richness and community structure of the gut microbiota and significantly enriched multiple bacterial taxa. Second, to isolate the role of obesity- and/or diet-influenced gut microbiota on CRC development, fecal microbial transplantation was performed by transferring the intestinal content from mice in the first study into recipient mice before chemical induction of CRC. We hypothesized that the gut microbiota from obese mice on obesogenic diets would promote CRC independent from the development of obesity. Our results indicated that gut microbiota shaped by the obesogenic diets was associated with worse colonic tumor measurements, while the differences in gut microbiota due to obesity or leanness did not affect CRC outcomes. Overall, we have demonstrated that diet and obesity have significant effects on gut microbial communities, but only dietary-induced gut microbial changes promote CRC. These results highlight the importance of understanding dietary effects on gut microbiota in CRC development which improves our ability to determine better strategies of prevention and treatment.

CHAPTER 1. INTRODUCTION

1.1 Colorectal cancer and obesity

1.1.1 Colorectal cancer prevalence and incidence

As of 2018, the Surveillance, Epidemiology, and End Results (SEER) Program at the National Cancer Institute (NCI) estimates that there are over 1.3 million men and women in the United States living with colorectal cancer (CRC), and that there will be almost 150,000 new cases and 53,000 deaths from this disease, with incidence and mortality higher in men than in women (1). CRC development is a slow process and is primarily diagnosed in individuals between the ages of 65 and 74. Data collected from the past 30 years have shown that the overall rate of new CRC cases and deaths has been steadily decreasing and the 5-year survival rate has been steadily increasing (2,3). These favorable trends are thought to be due changes in lifestyle factors such as decreased smoking and drinking, implementation of increased screening, and better methods of detection and treatment (4–6). Unfortunately, after stratifying by age, data from the past decade have shown that there has been an increase in CRC incidence in populations younger than 50 years old. These patients frequently present with an advanced stage of the disease (stage III/IV) compared to those 50 years and older (51.6% vs 40%) (7–10). Thus, continued research into mechanisms that contribute to CRC risk is important in identifying strategies that can help attenuate this unfortunate trend.

1.1.2 Initiation, progression, and promotion of colorectal cancer

First proposed in 2000 by Hanahan and Weinberg, the hallmarks of cancer delineate specific biological processes that occur in the initiation, progression, and promotion of cancer (11). As of 2022, there are eight hallmarks: sustained proliferative signaling, resisting cell death, evading growth suppressors, enabling replicative immortality, activating invasion and metastasis, sustained angiogenesis, avoiding immune destruction, and deregulating cellular metabolism; and two emerging hallmarks: unlocking phenotypic plasticity, and senescent cells. To address how cells develop these pro-tumorigenic capabilities they described four enabling characteristics of cancer: genome instability and mutation, tumor promoting inflammation, nonmutational

epigenetic programming, and polymorphic microbiomes (11–13).). Since at its core, the growth and survival of a tumor relies on cell proliferation, the most fundamental hallmark is arguably sustained proliferative signaling (12). Genetic changes that activate oncogenes (promote cell proliferation) and deactivate tumor suppressor genes (inhibits cell proliferation) are present in the initiation and throughout the progression of all types of cancer (12).

The genetic pathogenesis of CRC was first described by Fearon and Vogelstein. By analyzing the frequency of mutations present at each stage of the disease, they found that loss of the tumor suppressor gene, adenomatous polyposis coli (APC), was essential in the transformation of normal mucosa to small adenoma (polyp) (14,15). This gene is responsible for a protein that helps regulate the proliferative Wnt signaling pathway. In patients with familial adenomatous polyposis (FAP), a hereditary CRC caused by a germ line mutation in APC, risk of CRC is nearly 100% (16). Given the multistep process of tumorigenesis, the progression from adenoma to adenocarcinoma to carcinoma requires further genetic changes that affect other oncogenes and tumor suppressor genes such as K-RAS and TP53, respectively (17–19). Additional mutations that influence expression of cell-signaling pathways and proteins such as PI3K and SMAD4, cell-adhesion proteins such as N-cadherin, and DNA mismatch repair genes have also been shown to be important in tumor advancement (18,20–24). These genetic events are characteristic of the chromosomal instability pathway (CIN) of CRC development, which accounts for ~70% of sporadic cases (25). Other genetic pathways of CRC such as the microsatellite instability (MSI) pathway and the CpG island methylator phenotype (CIMP), account for ~15% and ~20-30%, respectively (24,26,27).

CRC arises from a complex interaction between genetic factors and environmental/lifestyles choices (28). Heredity forms of CRC such as familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC, also known as Lynch syndrome) compose 5-10% of CRC cases, and individuals with a family history of CRC make up ~20-30% of CRC cases. Most are sporadic (70-75%), meaning that there are no clear causes of the disease (18,29). Numerous environmental exposures and lifestyle habits have been linked to the development of CRC (3).

1.1.3 Mouse models of colorectal cancer

To study factors that contribute to CRC, researchers develop mouse models that are developed to emulate the progression of disease in humans. Since spontaneous CRC development in mice is rare, researchers have developed genetic and chemical models of CRC that include human-related attributes such as a loss of function mutation in APC, and tumor development in the colon and/or rectum (30,31).

A workhorse in CRC research is the $APC^{min/+}$ (multiple intestinal neoplasms) mouse. This model contains a germline mutation in the APC gene which causes the development of numerous small intestine tumors (32–34). Since most humans develop tumors in the colon and/or rectum, transgenic mice with a conditional gene targeting system, Cre-*lox*P- mediated recombination, are used to inactivate APC activity only in the colon and rectum (35). Researchers have developed other genetic models which have mutations that activate oncogenes or inactivate tumor suppressor genes. For example, mouse models with mutations in genes such as KRAS (36), p53 (37), SMAD3 (38) and TGFβ (39) have been created and used. Since transgenic mice can be costly to generate and maintain, many turn to a model of chemically-induced CRC (40,41).

There are a variety of chemical carcinogens used to model CRC such as 1,2-dimethylhydrazine (DMH), azoxymethane (AOM) (42), 2-amino-1-methyl-6-phenylimidozo [4,5-b] pyridine (PhIP) (43,44), N-Methyl-N-nitro-N-nitrosoguanidine (MNNG), and N-Methyl-N-nitrosourea (MNU) (45,46). A stable and reliable chemical used in hundreds of studies is AOM (31). AOM is administered over several weeks via subcutaneous or intraperitoneal injection and works in a dose dependent manner (42,47). In the liver, AOM is metabolized to methylazoxymethanol (MAM) via CYP2E1 and transported to the colon through bile or blood. AOM and MAM can damage DNA via alkylation or methylation, respectively (48,49). This method has been shown to target the Wnt signaling pathway, and to promote tumor development in the colon and/or rectum (50). However, success in AOM-induced CRC has been shown to be dependent on mouse strain, environment, and diet (42,51–54). For example, FVB/N, SWR, A/J have been shown to be susceptible to AOM induced colonic tumor development, while AKR/J, 129/SvJ and C57Bl/6J are not and instead develop preneoplastic lesions known as aberrant crypt foci (ACF) (52,53). Understanding ACF development provides insight into CRC initiation, but they can only be detected through techniques such as methylene staining of colon tissue (55–57).

In many strains, AOM-induced CRC develops faster after rounds of dextran sodium sulfate (DSS) ingestion, a chemical used to severely ulcerate and inflame the intestine (47,58,59).

While the models discussed above represent a small sampling of what is accessible, a single animal model has not yet been developed that is able to address the full extent of human CRC development. Both genetic and chemical models of CRC generally do not develop carcinomas or show signs of metastasis, and thus may be limited in addressing studies that examine more advanced stages of CRC (60). Those available are essential tools in assessing chemoprevention.

1.1.4 Obesity is a risk factor for colorectal cancer

One of the earliest cases of CRC was histologically identified in the mummified remains of an obese man, Ferrante I of Aragon, King of Naples (1431-1494 AD), whom due to his wealth and status had access to animal-based foods and indulgences that were high in sugar and fat (61). Unhealthy behaviors such as smoking, drinking and a sedentary lifestyle have been linked to CRC, and one well established risk factor is obesity (62–67). This phenomenon is modeled in mice, in which preneoplastic lesion development and colon tumor growth are enhanced by diet-induced obesity (68–72). Poor dietary patterns such as those high in fat and/or refined sugar, and processed meat and/or animal protein drive the development of obesity and have independently been implicated in promoting CRC (73–78). Some studies have found that obesity and increased consumption of sugar sweetened beverages are risk factors for early onset CRC (76,79,80). Numerous physiologic and metabolic changes that occur in obesity can contribute to CRC development such as increased inflammation, leptin, and insulin (81–90). As obesity incidence continues to rise in adults and adolescents in the United States, understanding potential mechanisms linking it to CRC is important in prevention and treatment (91,92).

1.1.5 Mouse models of obesity

Defining obesity encompasses a variety of anthropometric and biochemical measurements such as excess weight gain and body fat, and serum markers of inflammation, insulin resistance, and lipids (93). Some of these components have been shown to be adequately modeled in both genetic models of obesity and in diet-induced models of obesity. When using the latter, factors

such as diet composition, strain, age, and sex of the animal can significantly influence the success of the model.

Like humans, obesity in many rodents is driven by diets high in fat and sugar, also referred to as obesogenic diets. Reflecting the contribution of sugar sweetened beverages to the obesity epidemic (94), administering sugar in drinking water has been successfully used to induce obesity in rodents (95–100). However, using high concentrations of sucrose or fructose in a solid diet promotes obesity only when used in combination with high dietary fat (101–103). The cafeteria diet was formulated to model human consumption by giving animals free access to food such as salami and chocolate chips (96). However, with this design, it is difficult to accurately assess nutrient intake. Within the literature, obesity-inducing diets are frequently labeled as "high-fat" and "western", but there is no specific macronutrient profile assigned to either. Understanding exact diet composition is crucial as obesity development and related physiological and metabolic effects in mice have been shown to vary depending on aspects such as whether the lipid was composed of saturated fatty acids or unsaturated fatty acids (104–109). Semi-purified obesogenic diets are used to strictly control macronutrient source, type, and distribution. These products are offered by various companies and often contain between 40% and 60% fat from lard or milk. This percent range of saturated fat is significantly higher than the ~10% consumed by people in the United States, but in mice, it enables obesity to develop sooner (93,110). Depending on the exact diet composition, the development of obesity can take 2-4 months, and with more time, obesity-related conditions become more apparent (93,103,111,112).

Only certain strains of mice will gain excess weight and/or develop associated conditions such as systemic inflammation and insulin resistance (113,114). For example, AKR/J, DBA/2J and C57BL/6J have been shown to be responsive to diet induced obesity (115,116), while FVB/N and A/J mouse strain are not despite consuming more calories (117,118). C57BL/6J mice are frequently used since they develop obesity on various types of high fat and/or high sucrose diets (1). Other parameters such as age and sex of the animal influence the development of obesity. Studies using C57BL/6J mice have shown that young (4.25 months) male mice gain more weight on an obesity-inducing diet compared to a female counterpart, while old (11 months) female mice on the same obesity-inducing diet gain more weight relative to male counterparts (120,121).

Genetic modifications such as leptin-deficient *ob/ob* and leptin receptor deficient *db/db* represent another approach to study the development and consequences of obesity (122,123).

Leptin is a hormone secreted by adipose tissue that is involved in appetite regulation. In both models, loss of leptin signaling leads to hyperphagia and excess weight gain. While both also develop hyperinsulinemia, hyperglycemia, and dyslipidemia, only the *db/db* mouse develops diabetes (123). Through analysis of *ob/ob* and *db/db,* mice researchers saw that leptin played an integral role in the development of obesity and set about understanding methods to exploit it to benefit humans (124). However, they soon found that leptin was often significantly higher in obese patients relative to a lean control, and some proposed that obese individuals were resistant to leptin signaling rather than deficient in leptin (125). It is evident there are complex interactions between genetics and diet (126,127). Since one can more easily change their diet rather than their genetic code, and a benefit of using inbred strains of rodents is the elimination of genetic background as confounding factor, diet-induced obesity is a useful tool in studying the development of obesity and its role in mediating other chronic diseases.

1.1.6 Obesity-associated inflammation promotes a tumorigenic environment

Nineteenth century German pathologist, Rudolph Virchow first suggested the casual role of inflammation in cancer when he observed infiltration of leukocytes in neoplastic tissues (128). Inflammation describes a set of biological processes driven by immune cells responding to signals such as tissue damage, infection, cellular debris, and toxic compounds. Innate and adaptive immune cells can produce and secrete cytokines and chemokines that promote apoptotic pathways and recruit more immune cells to efficiently respond to a perceived threat (129). Once cleared, to promote tissue healing activities like cell proliferation, anti-inflammatory processes increase as pro-inflammatory processes decrease. Immune cells are polarized to different phenotypes to mediate these activities. For example, macrophages can differentiate to an M1-inflammatory phenotype or M2- anti-inflammatory phenotype, and T-cells can differentiate into the cytotoxic $CD8^+$ subtype or to a regulatory tolerogenic subtype (130). The equilibrium between cell death and cell proliferation is essential in maintaining homeostasis and preventing a tumorigenic environment (131,132). Loss of this balance through chronic inflammation can induce both apoptotic and proliferative pathways, increasing the likelihood for lasting DNA damage and subsequent tumor progression (130,133–135).

In obesity, many patients develop chronic systemic inflammation due in part to excess adipose tissue which acts not only as a place of energy storage, but also as a

paracrine/endocrine/autocrine tissue that can regulate metabolic and immunologic processes by secreting cytokines, chemokines, hormones and other signaling molecules (136). Between the two adipose depots, subcutaneous (under the skin) and visceral (surrounds organs), visceral is generally more inflammatory and is the major provider of such mediators (137–139). In obesity, this depot is populated by M1-like inflammatory macrophages that secrete cytokines such as TNF-α, and IL-1Ra, and IL-6 which can activate carcinogenic pathways via NFκB and STAT3 (140–148). These adipose tissue derived factors are secreted into circulation and can contribute to inflammation in the colon (149). In obesity, the colon is characterized by increased expression of inflammatory cytokines, production of reactive oxygen species, and proportion of inflammatory T-cells in the lamina propria (149–152). Separate from systemic factors, local factors in the colon such as the gut microbiota have been shown to be key mediators in intestinal health and in CRC initiation and progression (153–155).

1.2 The gut microbiota

1.2.1 A brief introduction to gut microbiota and disease

The human body is home to trillions of microbes. The majority reside in the colon where they synthesize vitamins, convert primary bile acids to secondary bile acids, protect against pathogenic infection, and are involved in host metabolism and immune modulation (156–160). Ninety-nine percent of the total population of intestinal microorganisms are bacteria (161). Within the bacterial kingdom, 90% fall under the Firmicutes and Bacteroidetes phyla, followed by Actinobacteria, Proteobacteria and Verrucomicrobia (162). Fecal microorganisms were observed as early as 1681 when Antoine van Leeuwenhoek examined his own stool underneath a microscope (163). A few hundred years later, as germ-theory was embraced by doctors, Theodor Escherich, Henry Tissier, Russian, Ilya Metchnikov and Alfred Nissle Escherich made great strides in understanding the relationship between bacteria and health and disease (164–170). More recently, interest in the gut microbiota grew in 2004 when Bäckhed et al. demonstrated that in mice, the gut microbiota had a casual role in the development of obesity (171,172). Large initiatives such as the Human Microbiome Project (HMP) and the Metagenomics of the Human Intestinal Tract consortium (MetaHIT) have since made significant progress in identifying, characterizing, and understanding the functions of the healthy human microbiota in part by sequencing the microbiome

(161,173,174). While there is tremendous interindividual variation in composition among humans, researchers have found that the gut microbiota of patients diagnosed with a disease, such as obesity or CRC, is different compared to their healthy counterparts, and the disease-associated microbiota is often referred to as dysbiosis (175–178). In animal studies, changing the number and types of microorganisms through diet, fecal microbial transplants (FMTs), or antibiotics was shown to attenuate or promote intestinal inflammation and/or colonic tumorigenesis (26,179–186). This evidence demonstrates that the gut microbiota is an important area of study in understanding potential strategies that help CRC prevention and treatment.

1.2.2 Mouse models used to study the gut microbiota

Studies have shown that the gut microbiota of humans and mice are both dominated by Bacteroidetes and Firmicutes and found that thirteen of the twenty top genera represented in mice were also represented in the top twenty of the humans (187–189). Although the mouse gut microbiome was functionally like humans', as determined by Kyoto Encyclopedia of Genes and Genomes (KEGG), only 4% of the mouse gut microbial genes were shared with those of the humans' (189). Other considerations such as differences in location of bacterial fermentation within the digestive tract are important. In mice, bacterial fermentation occurs primarily in the cecum, while in the human it occurs in the colon (187,190). Thus, comparisons of gut microbiota composition and metabolites with other studies, or inference about potential effects on disease phenotype depend on sample origin and species. Despite these differences, mouse models used to determine effects of the gut microbiota in disease development are critical in proof-of-concept studies, which was demonstrated by Patrice Cani's research group in their use of *Akkermansia muciniphilia* in the alleviation of obesity and related metabolic conditions in mice then humans (191,192).

One of the most significant advancements in gut microbiota research was the development of the germ-free mouse, which are those devoid of all microorganisms. In 1885, Louis Pasteur was the first documented scientist to conceive the notion of a germ-free animal, but it wasn't until 1959 when Julian R. Pleasants produced germ free mice (193). This model allows researchers to understand disease in the complete absence of microbes or in the presence of specific microbes, which are known as gnotobiotic mice. Although germ-free mice are impaired in aspects of development and immune system maturation, they allow researchers to propose a causal link

between specific microorganisms and a disease (194). Unfortunately, germ-free mice require special care and maintenance that is costly. An alternative is to use antibiotic treated conventional mice, which are those with a natural gut microbiota (195). In this model, the gut microbiota of conventional mice is depleted after treatment with a broad-spectrum bactericidal cocktail that often includes ampicillin, neomycin, metronidazole, and vancomycin (196,197). This cocktail can be administered through drinking water or oral gavage, or a combination of the two. Treatment through drinking water is less cumbersome, but dosing is more imprecise. Oral gavage ensures equal dosing, but can be a stressful process for the mouse, especially when protocols call for multiple administrations over a many weeks-long period (198). Antibiotic treatments have been shown to disturb colonic homeostasis, but this method is a more attainable option for many researchers and in many studies (198,199).

Using these mouse models to understand the effect of gut microbiota on disease progression often requires a fecal microbial transplant (FMT). This is a procedure where fecal and/or cecal samples are first collected then stored in an -80°C freezer or used directly for transplantation into recipient mice, often via oral gavage. There are numerous variations on each step that can influence results (200). For example, samples may be processed in open air which can affect the viability of anaerobic bacteria, or the FMT can be administered by an enema, bypassing the gastrointestinal tract (201). In addition, since mice are social creatures and display coprophagy, FMT can be done through cohousing mice. The frequency of FMT in a study is another important variable (200). In one experiment, researchers found that after one FMT, donor microbiota was still detected in recipients after 4 weeks, but there was evidence that the composition was shifting over time (202). Variations within these methods can make studies difficult to replicate, and unfortunately, gut microbiota composition is also affected by mouse strain, mouse vendor, housing conditions, diet source, and other environmental factors that can be hard to control (187,203,204). As with any animal model, translation to humans is not direct, but these methods have proven to be integral in better understanding the gut microbiota in disease progression.

1.2.3 Bacteria and intestinal homeostasis

The colon is an essential physical barrier that controls the movement of luminal contents into the body. The colonic mucosa is composed of crypts, finger-like invaginations composed of

stem cells, goblet cells and colonocytes, and the lamina propria, which is connective tissue that contains immune cells and vasculature for blood and lymph (205,206). The luminal-mucosa juncture is a dynamic microenvironment where bacteria, nutrients, colonocytes, and immune cells interact with each other to maintain homeostasis. Commensal microbes have a protective role in the colon. For example, toll-like receptors (TLRs) or nucleotide-binding oligomerization domain molecules (NODs) expressed by dendritic cells, macrophages and colonocytes respond to bacteria with anti-inflammatory responses that help protect against intestinal injury (207,208). Although the presence of non-host cells like bacteria could incite an inflammatory response, commensal microbes have been shown to promote tolerogenic T-cell differentiation and expansion, increased intestinal macrophage polarization to the M2 anti-inflammatory phenotype, and correct underdeveloped immune systems (209–212). Colonic health is also characterized by the ability to adequately respond to inflammatory stimuli. In mice, a loss or weakening of pathways known to induce an inflammatory response, were more susceptible to chemically induced intestinal damage and pathogenic infection (207,213–215). Complementary to microbiota influencing intestinal mucosa, changes in or to mucosal cells such as loss or constitutive expression of colon specific TLR4, treatment with non-steroidal anti-inflammatory drugs, and shifts in colonocyte metabolism have been shown to mediate changes in gut microbiota composition (216–218).

There are numerous mechanisms by which microbiota influence the intestinal environment and vice versa, and one well-documented method is through bacterial metabolites (219). In 2018, Litvek et al introduced the concept of the C1/C2 colonocyte, an extension of the M1/M2 macrophage categorization. Polarization to anti-inflammatory C2 was dependent on activation of PPARγ by the microbially produced short chain fatty acid (SCFA), butyrate (216,220). The PPARγ nuclear receptor is highly expressed in the colon. Signaling through PPARγ increased beta-oxidation, which helped to maintain the hypoxic colonic environment (220,221). This in turn was associated with decreased presence of facultative anaerobes like Proteobacteria, which are commonly measured in disease (222). Reduced intestinal oxygenation is important in stabilization of the transcription factor, hypoxia inducible factor (HIF), whose target genes work to maintain barrier integrity (223), microbial defense (224), and mucus production (225). In addition, PPARγ activation has been associated with positive effects on intestinal barrier function, and inhibited CRC by promoting growth arrest, cell differentiation, and decreased tumor growth (226–230). There are a multitude of complex and intricate relationships among microbes and the intestine

(231–233). For example, microbial metabolites also provide substrate for other microbes in a process known as cross-feeding (234). The summation of these interactions is important in understanding the mechanisms by which microbes influence colonic health.

Changes in gut microbiota composition that disturb homeostatic mechanisms can increase inflammation and promote a tumorigenic environment in the colon (235–237). Various factors influence the gut microbiota such as age, environment, host genetics, and antibiotic use. However, as undigested nutrients in the colon provide substrate for intestinal microorganisms, diet is the primary contributor (238–246).

1.2.4 Assessing gut microbiota composition

There are different sequencing technologies that allow researchers to assess the gut microbiota. In metagenomics, all microbial genes are sequenced, collectively known as the gut microbiome. This provides information not only on microbial community, but also on functional and metabolic diversity. However, this can be expensive and computationally intensive. One of the most common, less expensive methods to assess gut microbial communities is through targeted 16S ribosomal RNA (rRNA) gene sequencing. This gene is an evolutionarily conserved component of the prokaryotic ribosome 30S subunit that is present in almost all bacteria, making it an ideal taxonomic and phylogenic marker (204,247–250). Researchers can distinguish between different bacteria due to variations in hypervariable regions (V1-V9) that are interspersed with conserved regions (251). Bacterial identification from 16S rRNA gene sequencing provides a basic assessment of microbial community structure. Sequences generated through this method are termed amplicon sequence variants (ASVs) and are the basis for measuring microbial community structure and composition. Although amplicon sequence variant is a more precise and correct term, for ease, taxon/taxa will be used. Alpha and beta diversity are used to summarize the richness and relative abundance of taxa in a sample and to calculate microbial community dissimilarities between two samples, respectively. There are a variety of metrics used to calculate each type of diversity.

In alpha diversity, observed taxonomic units and Faith's PD are measurements of taxa richness in a community. However, observed features is a count of the number of unique sequences present, while Faith's PD considers phylogenetic relationships and is a summation of branch lengths of the phylogenetic tree (252). Abundance can be described using Pielou's evenness, which

is an assessment of how evenly taxa are distributed in a sample on a scale of 0 (no evenness) to 1 (perfectly even) (253). Another alpha diversity metric, Shannon Entropy integrates both community richness and abundance (254).

In beta-diversity, Bray-Curtis dissimilarity and Jaccard index calculate a statistic that quantifies the dissimilarity or similarity, respectively between two microbial communities (255,256). Both metrics are scored from zero to one. For Bray-Curtis, zero indicates two samples share all taxa and one indicates two samples share no taxa, and for Jaccard, zero indicates two samples share no taxa and one indicates two samples share all taxa. Another method is UniFrac, which was designed to assess differences between microbial communities (257,258). UniFrac is a distance metric used to define dissimilarity between microbial communities while incorporating phylogenetic relationships (257). In weighted UniFrac, the distance accounts for abundance of taxa while in unweighted UniFrac, the distance only considers presence and absence of taxa.

Alongside overall microbial community structure, assigning sequences to specific taxa names is also important in assessing the gut microbiota. This is done through comparison with a database of sequences such as SILVA (259), Greengenes (260) or Ribosomal Database Project (RDP) (261,262). There are differences among databases in terms of coverage and frequency of update. Choice of alpha diversity, beta diversity, and taxonomic classification methods can significantly influence results. There is no golden standard for analysis so many researchers calculate numerous alpha and beta diversity metrics and/or compare taxonomy across different databases to gain confidence in the results.

1.2.5 Effects of a westernized diet on the gut microbiota influence the colonic environment

Many studies have measured shifts in gut microbiota composition in response to changes in diet (241,263,264). The gut microbiome is also influenced by diet. In a study comparing omnivore, vegetarian, and vegan gut microbiomes, vegetarian and vegan microbiomes were enriched for genes responsible for carbohydrate and amino acid metabolism, synthesis of essential amino acids and vitamins, and cell motility (264). Metagenomic sequencing has revealed many microbial enzymes, but most are not characterized or well-understood (265). Regardless, the presence of the gut microbiome provides humans with the benefits and detriments of microbial metabolites. A westernized diet low in fiber and high in saturated fat and animal protein is

associated with CRC and may be in part because bacterial metabolism of fiber, amino acids and lipids have significant implications in colonic health and CRC risk (266–269).

Much research has focused on microbial fermentation of fiber, more specifically microbiota accessible carbohydrates (MACS), due to their association with decreased risk for disease and indigestibility by humans and thus, increased availability to microbes (266,270,271). Microbial degradation of fiber produces simple sugars that can be further metabolized to short chain fatty acids (SCFAs) like acetate, propionate, and butyrate. Although butyrate makes up only 15% of all SCFAs, it provides colonocytes with 70% of their energy needs (272). A healthy gut microbiota is associated with increased butyrate and butyrate producers such as *Faecalibacterium prausnitzii*, *Roseburia intestinalis*, *Eubacterium rectale, and Anaerostipes butyraticus* (175,273–275). This SCFA imparts its beneficial effects in colonic homeostasis and CRC by influencing colonocyte proliferation, differentiation, apoptosis, and intestinal barrier integrity (276–279). In addition, butyrate was shown to influence gene expression in immune cells to promote immunosuppressive phenotypes such as Foxp3+ regulatory T-cells and dendritic cells that suppress naïve T-cell maturation into inflammatory IFN-γ producing T-cells (280–284). These results underly the potential positive effects of microbiota and fiber in preventing CRC (267,285).

A mechanism linking high protein diets to increased CRC risk is bacterial production of hydrogen sulfide (H_2S) from sulfur-containing amino acids such as methionine and cysteine (286). RNA-seq analysis of fecal samples from human participants consuming a high protein diet showed a significant increase in sulphite reductase expression. These are enzymes that reduce sulfate to elemental sulfur, the form used by bacteria to produce hydrogen sulfide (H_2S), a genotoxic compound that can increase intestinal inflammation and impair butyrate oxidation (287–293). Sulfate reducing bacteria such as *Bilophila wadsworthia,* and *Fusobacterium nucleatum* have been shown to be enriched in CRC patients compared to controls (294–297). However, non-animal based protein such as whey and pea protein have been reported to promote the growth of commensal *Bifidobacterium* and *Lactobacillus* alongside an increase in acetate, propionate and butyrate, and a decrease in sulfite reducing *Clostridia* (298,299). These differences based on protein source highlight the importance of diet in mediating gut microbiota composition and function.

Lipids can interact with and influence the microbiota by providing substrate and by increasing bile acid secretion. The glycerol backbone of triglycerides can be metabolized to 1,3-

propanediol by certain species of Proteobacteria, *Clostridia, Lactobacilli,* and *Enterococci* (300). During this process reuterin (3-hydroxypropanol) is produced (301). Reuterin has been shown to promote inflammation and oxidative stress, and can be spontaneously converted to acrolein, a reactive genotoxin, which can increase risk for DNA damage (301,302). Since lipids are efficiently digested and absorbed, they can also influence the colonic environment through their role in stimulating bile acid secretion. Bile acids are essential in emulsifying lipids to aid in fat digestion and absorption (303). Although most bile acids are reabsorbed, those that pass to the colon can be acted on by bacterial 7α-dehydroxylase which converts primary bile acids into secondary bile acids (304,305). High concentrations of bile acids in the colon are linked to oxidative DNA damage, cell membrane distress, decrease in DNA repair enzymes and other cell stressors that promote genomic instability (306). CRC patients are shown to have high fecal and serum levels of secondary bile acids such as deoxycholic acid (DCA) (307–309). In animal models and cell models of CRC, DCA supplementation was shown to be associated with low grade inflammation, impaired intestinal permeability, resistance to apoptosis, and increased tumor number (310–313).

Diet can clearly influence gut microbiota to mediate colonic health and CRC risk. In mice, obesity-inducing diets such as those high in fat or high in fat and sugar have been shown to disturb intestinal homeostasis by weakening barrier function, decreasing mucus production and increasing and exacerbating colonic inflammation (151,180,314–319). Although these models are also associated with changes to the gut microbiota (152,180,320,321), it is possible that in addition to diet, obesity-related conditions such as increased systemic inflammation and circulating growth factors can have an impact. Using mouse models resistant to obesity, previous research has demonstrated that diet is the primary driver of gut microbiota composition, independent from the development of obesity (320,321). However, genotype was the second major predictor, likely masking any effects of obesity and preventing comparison to an obese counterpart. In a recently published study, researchers demonstrated that systemic and colonic inflammation in mice on a high fat diet or high sucrose diet was transferrable via fecal microbial transplant (FMT). However, in this study, mice on the high fat diet gained more weight than mice on the high sucrose diet, so the effect of obesity-related factors on gut microbiota could have confounded these results. In addition, the FMTs were performed four times a week for four weeks, which is a stressful process that can induce inflammation. From their methods, it appeared that there was no FMT given to their control group, thus there was no control for the inflammatory effects due to the FMT process

(322). Other mouse studies that used prebiotics, probiotics, and antibiotics to modulate the gut microbiota of obese mice have shown positive effects on inflammation, but these treatments also led to weight and fat loss (180,323,324). Given the intimate and complex relationship between diet, obesity, and the gut microbiota, it is important to tease out individual effects to better understand the role of the gut microbiota in obesity-associated CRC risk.

1.3 Obesity, gut microbiota, and colorectal cancer

1.3.1 Gut microbiota and colorectal cancer

The first report directly implicating the gut microbiota in CRC was published in 1975. In this study, researchers demonstrated that in a chemically induced model of CRC, germ-free mice developed fewer tumors than those conventionally raised (325). These results have since been replicated in germ-free rats (326) and in genetic models of CRC (327). Since then, other researchers have demonstrated the impact of bacteria-mediated inflammation on the risk and progression of CRC. In models of inflammation induced tumorigenesis, using antibiotics, or inhibiting TLR4 was associated with reduced tumor number and decreased colonic macrophages and inflammatory cytokine expression (179,184,185).

Within the microbiota-CRC literature, researchers have identified bacteria linked to CRC development via specific mechanisms. Three bacteria that have been well studied are *Fusobacterium nucleatum,* enterotoxigenic *Bacteroides fragilis* (ETBF)*,* and *Escherichia coli* expressing polyketide synthase (*E. coli* pks+) (155). All three bacteria have been shown to be enriched in fecal samples from patients with CRC relative to healthy controls (295,296,328) and in adenoma tissue compared to adjacent normal tissue (329,330). These bacteria exert their effects in various ways such as by producing genotoxins like colibactin (*E. coli)* or by increasing β−catenin signaling to promote cell proliferation (*F. nucleatum)* and by activating the STAT3/IL-17 immune response to increase local inflammation (ETBF) (296,331–333). In a genetic model of CRC, *F. nucleatum* and *B. fragilis* promoted tumor infiltrating myeloid cells and accelerated the development and increased the number of tumors (334). This inflammatory signature was comparable to those measured in human CRCs positive for *F. nucleatum* (295). However, these microbes alone do not significantly influence CRC development. For example, the presence of *E. coli* pks+ increases colon tumor development only in the presence of inflammation, such as in an

IL10$^{-/-}$ mouse model, highlighting the necessity of inflammation or other stimuli in bacterially mediated promotion of CRC (332). While learning the mechanisms of specific microbes in CRC initiation and progression lend insight into potentially exploitable pathways for prevention and treatment, given the complexity of the microbial ecosystem present in humans, it is also necessary to understand how a community of gut microbes affect this disease.

Various analyses have demonstrated how human CRC patients have distinct gut microbiotas compared to their healthy counterparts (176,335–337). In both genetic and chemically induced models of CRC, mice that received a fecal microbial transplant from CRC patients (FMT-CRC) developed more tumors and had decreased apoptosis compared to mice receiving healthy control FMT (FMT-H) (177,338). The colons of FMT-CRC mice also had higher levels of inflammatory cytokines like IL-1β and TNF-α, alongside decreased tight junction protein expression and mucus production (Muc2). These results suggested that FMT-CRC increased colonic inflammation to promote CRC development (177). In obesity, there are similar signs of increased colonic inflammation that may mediate CRC development, but it is difficult to determine effects due only to the gut microbiota given the presence of obesity related chronic inflammation.

1.3.2 Obesity-associated gut microbiota in colorectal cancer development

A few studies have assessed the role of obesity-associated gut microbiota independent from the physiological setting of obesity. Using FMTs and antibiotics, these studies found that found that high fat diet impacted the gut microbiota to promote CRC independent from the development of obesity (339–341). However, these studies relied on a small intestine model of cancer, which has a significantly different gut microbiota compared to the colon, where human CRC develops (342,343). By using a mouse model that was resistant to diet-induced obesity or using a model that developed obesity, researchers were limited in their conclusions about the role of diet and obesity mediating CRC by modulating the gut microbiota. Further elucidation of this relationship may help uncover areas of intervention that will help weaken the strong association between obesity and increased risk for CRC.

1.4 Summary

The development of CRC generally occurs in older populations because it is a multistep process mediated by mutations in oncogenes and tumor suppressor genes. These potential tumorigenic cells are normally corrected or cleared to prevent dysregulated cell proliferation. Unfortunately, CRC incidence is rising in younger populations, suggesting that there are lifestyle and/or environmental factors that may be responsible for increasing the rate of this disease. While there are a variety of components that can mediate this link, obesity has been consistently associated with increased risk for the development of CRC and is on the rise in adolescents and adults. Obesity is a complex condition that promotes various pro-tumorigenic process such as increased inflammation, growth factors, and hormones that stem in large part from excess adipose tissue. In the colon, a more local source of potential pro-tumorigenic processes is the gut microbiota.

Colonic bacteria have been shown to be important in maintaining intestinal homeostasis by modulating immune cells and intestinal epithelial cells. Obesity associated changes to the gut microbiota have been shown to disturb the balance between pro and anti-inflammatory processes in the colon, which can increase the risk for cancer development. There is a plethora of factors that influence gut microbiota composition including host genetics, but as intestinal microbes rely on dietary substrates that are in the colon, diet is a major contributor. Since diet is also a primary driver of obesity, the composition of obesogenic diets may have some leverage in impacting microbial effects in tumorigenesis. Previous research has demonstrated that changes to the gut microbiota can have significant effects in CRC risk, so is it important to learn if and to what extent obesity and/or obesogenic diets influence gut microbiota-mediated CRC development. Considering most living humans eat, diet is an ideal target in CRC prevention. Better understanding of the effect of various dietary patterns on the gut microbiota will help direct effective and sustainable strategies that may help to decrease the rising rate of CRC incidence in young adults.

1.5 References

1. National Cancer Institute. Seer Training Modules, Colorectal Cancer, Abstracting, Coding, & Staging, Abstract Keys [Internet]. Available from: https://training.seer.cancer.gov/colorectal/abstract-code-stage/keys.html

2. Surveillance, Epidemiology, and End Results Program. https://seer.cancer.gov/statfacts/html/colorect.html [Internet]. Cancer Statistics. [cited 2021 Nov 9]. Available from: https://seer.cancer.gov/statfacts/html/colorect.html

3. American Cancer Society. Colorectal Cancer Facts & Figures 2020-2022. 2020.

4. Durko L, Malecka-Panas E. Lifestyle Modifications and Colorectal Cancer. Curr Colorectal Cancer Rep. 2014 Mar;10(1):45–54.

5. Gingras D, Béliveau R. Colorectal Cancer Prevention Through Dietary and Lifestyle Modifications. Cancer Microenviron. 2011 Aug;4(2):133–9.

6. Derry MM, Raina K, Agarwal C, Agarwal R. Identifying Molecular Targets of Lifestyle Modifications in Colon Cancer Prevention. Front Oncol [Internet]. 2013 [cited 2022 Mar 14];3. Available from: http://journal.frontiersin.org/article/10.3389/fonc.2013.00119/abstract

7. Virostko J, Capasso A, Yankeelov TE, Goodgame B. Recent trends in the age at diagnosis of colorectal cancer in the US National Cancer Data Base, 2004-2015. Cancer. 2019 Nov;125(21):3828–35.

8. Siegel RL, Miller KD, Goding Sauer A, Fedewa SA, Butterly LF, Anderson JC, et al. Colorectal cancer statistics, 2020. CA Cancer J Clin. 2020 May;70(3):145–64.

9. Brenner DR, Heer E, Sutherland RL, Ruan Y, Tinmouth J, Heitman SJ, et al. National Trends in Colorectal Cancer Incidence Among Older and Younger Adults in Canada. JAMA Netw Open. 2019 Jul 31;2(7):e198090.

10. Araghi M, Soerjomataram I, Bardot A, Ferlay J, Cabasag CJ, Morrison DS, et al. Changes in colorectal cancer incidence in seven high-income countries: a population-based study. Lancet Gastroenterol Hepatol. 2019 Jul;4(7):511–8.

11. Hanahan D, Weinberg RA. The Hallmarks of Cancer. Cell. 2000 Jan;100(1):57–70.

12. Hanahan D, Weinberg RA. Hallmarks of Cancer: The Next Generation. Cell. 2011 Mar;144(5):646–74.

13. Hanahan D. Hallmarks of Cancer: New Dimensions. Cancer Discov. 2022 Jan;12(1):31–46.

14. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. Cell. 1990 Jun;61(5):759–67.

15. Fearnhead NS. The ABC of APC. Hum Mol Genet. 2001 Apr 1;10(7):721–33.

16. Half E, Bercovich D, Rozen P. Familial adenomatous polyposis. Orphanet J Rare Dis. 2009 Dec;4(1):22.

17. Fearon ER. Molecular Genetics of Colorectal Cancer. Annu Rev Pathol Mech Dis. 2011 Feb 28;6(1):479–507.

18. Yamagishi H, Kuroda H, Imai Y, Hiraishi H. Molecular pathogenesis of sporadic colorectal cancers. Chin J Cancer. 2016 Jan 6;35:4.

19. Smith G, Carey FA, Beattie J, Wilkie MJV, Lightfoot TJ, Coxhead J, et al. Mutations in APC, Kirsten-ras, and p53—alternative genetic pathways to colorectal cancer. Proc Natl Acad Sci. 2002 Jul 9;99(14):9433–8.

20. Yang J, McDowell A, Kim EK, Seo H, Lee WH, Moon C-M, et al. Development of a colorectal cancer diagnostic model and dietary risk assessment through gut microbiome analysis. Exp Mol Med. 2019 Oct;51(10):1–15.

21. Armaghany T, Wilson JD, Chu Q, Mills G. Genetic alterations in colorectal cancer. Gastrointest Cancer Res GCR. 2012 Jan;5(1):19–27.

22. Pećina-Šlaus N, Kafka A, Salamon I, Bukovac A. Mismatch Repair Pathway, Genome Stability and Cancer. Front Mol Biosci. 2020 Jun 26;7:122.

23. Wheeler JMD. DNA mismatch repair genes and colorectal cancer. Gut. 2000 Jul 1;47(1):148–53.

24. Nguyen H, Duong H. The molecular characteristics of colorectal cancer: Implications for diagnosis and therapy (Review). Oncol Lett [Internet]. 2018 May 9 [cited 2022 Jan 31]; Available from: http://www.spandidos-publications.com/10.3892/ol.2018.8679

25. Worthley DL, Leggett BA. Colorectal cancer: molecular features and clinical opportunities. Clin Biochem Rev. 2010 May;31(2):31–8.

26. De Palma G, Lynch MDJ, Lu J, Dang VT, Deng Y, Jury J, et al. Transplantation of fecal microbiota from patients with irritable bowel syndrome alters gut function and behavior in recipient mice. Sci Transl Med. 2017 Mar 1;9(379):eaaf6397.

27. Colussi D, Brandi G, Bazzoli F, Ricciardiello L. Molecular Pathways Involved in Colorectal Cancer: Implications for Disease Behavior and Prevention. Int J Mol Sci. 2013 Aug 7;14(8):16365–85.

28. Rustgi AK. The genetics of hereditary colon cancer. Genes Dev. 2007 Oct 15;21(20):2525–38.

29. Jasperson KW, Tuohy TM, Neklason DW, Burt RW. Hereditary and Familial Colon Cancer. Gastroenterology. 2010 May;138(6):2044–58.

30. Rowlatt C, Franks LM, Sheriff MU, Chesterman FC. Naturally occurring tumors and other lesions of the digestive tract in untreated C57BL mice. J Natl Cancer Inst. 1969 Dec;43(6):1353–64.

31. Rosenberg DW, Giardina C, Tanaka T. Mouse models for the study of colon carcinogenesis. Carcinogenesis. 2008 Oct 8;30(2):183–96.

32. Moser AR, Pitot HC, Dove WF. A Dominant Mutation That Predisposes to Multiple Intestinal Neoplasia in the Mouse. Science. 1990 Jan 19;247(4940):322–4.

33. Washington K, Zemper AED. Apc-related models of intestinal neoplasia: a brief review for pathologists. Surg Exp Pathol. 2019 Dec;2(1):11.

34. Luongo C, Moser AR, Gledhill S, Dove WF. Loss of Apc+ in intestinal adenomas from Min mice. Cancer Res. 1994 Nov 15;54(22):5947–52.

35. Shibata H, Toyama K, Shioya H, Ito M, Hirota M, Hasegawa S, et al. Rapid Colorectal Adenoma Formation Initiated by Conditional Targeting of the *Apc* Gene. Science. 1997 Oct 3;278(5335):120–3.

36. Boutin AT, Liao W-T, Wang M, Hwang SS, Karpinets TV, Cheung H, et al. Oncogenic *Kras* drives invasion and maintains metastases in colorectal cancer. Genes Dev. 2017 Feb 15;31(4):370–82.

37. Harvey M, McArthur MJ, Montgomery CA, Butel JS, Bradley A, Donehower LA. Spontaneous and carcinogen–induced tumorigenesis in p53–deficient mice. Nat Genet. 1993 Nov;5(3):225–9.

38. Zhu Y, Richardson JA, Parada LF, Graff JM. Smad3 Mutant Mice Develop Metastatic Colorectal Cancer. Cell. 1998 Sep;94(6):703–14.

39. Engle SJ, Hoying JB, Boivin GP, Ormsby I, Gartside PS, Doetschman T. Transforming growth factor beta1 suppresses nonmetastatic colon cancer at an early stage of tumorigenesis. Cancer Res. 1999 Jul 15;59(14):3379–86.

40. Kim H, Kim M, Im S-K, Fang S. Mouse Cre-LoxP system: general principles to determine tissue-specific roles of target genes. Lab Anim Res. 2018 Dec;34(4):147–59.

41. Orban PC, Chui D, Marth JD. Tissue- and site-specific DNA recombination in transgenic mice. Proc Natl Acad Sci. 1992 Aug;89(15):6861–5.

42. Bissahoyo A, Pearsall RS, Hanlon K, Amann V, Hicks D, Godfrey VL, et al. Azoxymethane Is a Genetic Background-Dependent Colorectal Tumor Initiator and Promoter in Mice: Effects of Dose, Route, and Diet. Toxicol Sci. 2005 Dec 1;88(2):340–5.

43. Tudek B, Bird RP, Bruce WR. Foci of aberrant crypts in the colons of mice and rats exposed to carcinogens associated with foods. Cancer Res. 1989 Mar 1;49(5):1236–40.

44. Esumi H, Ohgaki H, Kohzen E, Takayama S, Sugimura T. Induction of Lymphoma in CDF $_1$ Mice by the Food Mutagen, 2-Amino-l-methyl-6-phenylimidazo[4,5- *b*]pyridine. Jpn J Cancer Res. 1989 Dec;80(12):1176–8.

45. Jobst K. Teratogenous changes and tumors in rats following treatment with methylnitroso-urea (MNU). Neoplasma. 1967;14(4):435–6.

46. Ito N, Hasegawa R, Imaida K, Tamano S, Hagiwara A, Hirose M, et al. Carcinogenicity of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in the rat. Mutat Res Mol Mech Mutagen. 1997 May;376(1–2):107–14.

47. Neufert C, Becker C, Neurath MF. An inducible mouse model of colon carcinogenesis for the analysis of sporadic and inflammation-driven tumor progression. Nat Protoc. 2007 Aug;2(8):1998–2004.

48. Nyskohus LS, Watson AJ, Margison GP, Le Leu RK, Kim SW, Lockett TJ, et al. Repair and removal of azoxymethane-induced O6-methylguanine in rat colon by O6-methylguanine DNA methyltransferase and apoptosis. Mutat Res Toxicol Environ Mutagen. 2013 Dec;758(1–2):80–6.

49. Sohn OS, Fiala ES, Requeijo SP, Weisburger JH, Gonzalez FJ. Differential effects of CYP2E1 status on the metabolic activation of the colon carcinogens azoxymethane and methylazoxymethanol. Cancer Res. 2001 Dec 1;61(23):8435–40.

50. Yamada Y, Mori H. Multistep carcinogenesis of the colon in Apc $^{Min/+}$ mouse. Cancer Sci. 2007 Jan;98(1):6–10.

51. Diwan BA, Blackman KE. Differential susceptibility of 3 sublines of C57BL6 mice to the induction of colorectal tumors by 1,2-dimethylhydrazine. Cancer Lett. 1980 Apr;9(2):111–5.

52. Papanikolaou A, Shank RC, Delker DA, Povey A, Cooper DP, Rosenberg DW. Initial levels of azoxymethane-induced DNA methyl adducts are not predictive of tumor susceptibility in inbred mice. Toxicol Appl Pharmacol. 1998 May;150(1):196–203.

53. Nambiar PR, Girnun G, Lillo NA, Guda K, Whiteley HE, Rosenberg DW. Preliminary analysis of azoxymethane induced colon tumors in inbred mice commonly used as transgenic/knockout progenitors. Int J Oncol. 2003 Jan;22(1):145–50.

54. Shitashige M, Satow R, Honda K, Ono M, Hirohashi S, Yamada T. Increased susceptibility of Sf1 $^{+/-}$ mice to azoxymethane-induced colon tumorigenesis. Cancer Sci. 2007 Dec;98(12):1862–7.

55. Bird RP. Role of aberrant crypt foci in understanding the pathogenesis of colon cancer. Cancer Lett. 1995 Jun;93(1):55–71.

56. Ochiai M, Watanabe M, Nakanishi M, Taguchi A, Sugimura T, Nakagama H. Differential staining of dysplastic aberrant crypt foci in the colon facilitates prediction of carcinogenic potentials of chemicals in rats. Cancer Lett. 2005 Mar;220(1):67–74.

57. McGinley JN, Thompson MD, Thompson HJ. A Method for Serial Tissue Processing and Parallel Analysis of Aberrant Crypt Morphology, Mucin Depletion, and Beta-Catenin Staining in an Experimental Model of Colon Carcinogenesis. Biol Proced Online. 2010 Dec;12(1):118.

58. Fazio V, Robertis M, Massi E, Poeta M, Carotti S, Morini S, et al. The AOM/DSS murine model for the study of colon carcinogenesis: From pathways to diagnosis and therapy studies. J Carcinog. 2011;10(1):9.

59. Suzuki R, Kohno H, Sugie S, Nakagama H, Tanaka T. Strain differences in the susceptibility to azoxymethane and dextran sodium sulfate-induced colon carcinogenesis in mice. Carcinogenesis. 2005 Jan 1;27(1):162–9.

60. Bürtin F, Mullins CS, Linnebacher M. Mouse models of colorectal cancer: Past, present and future perspectives. World J Gastroenterol. 2020 Apr 7;26(13):1394–426.

61. Fornaciari G. Malignant tumor in the mummy of Ferrante Ist of Aragon, King of Naples (1431-1494). Med Secoli. 1994;6(1):139–46.

62. Moghaddam AA, Woodward M, Huxley R. Obesity and Risk of Colorectal Cancer: A Meta-analysis of 31 Studies with 70,000 Events. Cancer Epidemiol Biomarkers Prev. 2007 Dec 1;16(12):2533–47.

63. Abar L, Vieira AR, Aune D, Sobiecki JG, Vingeliene S, Polemiti E, et al. Height and body fatness and colorectal cancer risk: an update of the WCRF–AICR systematic review of published prospective studies. Eur J Nutr. 2018 Aug;57(5):1701–20.

64. Larsson SC, Wolk A. Obesity and colon and rectal cancer risk: a meta-analysis of prospective studies. Am J Clin Nutr. 2007 Sep 1;86(3):556–65.

65. Le Marchand L, Wilkens LR, Kolonel LN, Hankin JH, Lyu LC. Associations of sedentary lifestyle, obesity, smoking, alcohol use, and diabetes with the risk of colorectal cancer. Cancer Res. 1997 Nov 1;57(21):4787–94.

66. Johnson CM, Wei C, Ensor JE, Smolenski DJ, Amos CI, Levin B, et al. Meta-analyses of colorectal cancer risk factors. Cancer Causes Control. 2013 Jun;24(6):1207–22.

67. Sinicrope FA, Foster NR, Sargent DJ, O'Connell MJ, Rankin C. Obesity Is an Independent Prognostic Variable in Colon Cancer Survivors. Clin Cancer Res. 2010 Mar 15;16(6):1884–93.

68. O'Neill AM, Burrington CM, Gillaspie EA, Lynch DT, Horsman MJ, Greene MW. High-fat Western diet–induced obesity contributes to increased tumor growth in mouse models of human colon cancer. Nutr Res. 2016 Dec;36(12):1325–34.

69. Olivo-Marston SE, Hursting SD, Perkins SN, Schetter A, Khan M, Croce C, et al. Effects of calorie restriction and diet-induced obesity on murine colon carcinogenesis, growth and inflammatory factors, and microRNA expression. PloS One. 2014;9(4):e94765.

70. Rondini EA, Harvey AE, Steibel JP, Hursting SD, Fenton JI. Energy balance modulates colon tumor growth: Interactive roles of insulin and estrogen. Mol Carcinog. 2011 May;50(5):370–82.

71. Day SD, Enos RT, McClellan JL, Steiner JL, Velázquez KT, Murphy EA. Linking inflammation to tumorigenesis in a mouse model of high-fat-diet-enhanced colon cancer. Cytokine. 2013 Oct;64(1):454–62.

72. Gravaghi C, Bo J, LaPerle KMD, Quimby F, Kucherlapati R, Edelmann W, et al. Obesity enhances gastrointestinal tumorigenesis in Apc-mutant mice. Int J Obes. 2008 Nov;32(11):1716–9.

73. Liao LM, Loftfield E, Etemadi A, Graubard BI, Sinha R. Substitution of dietary protein sources in relation to colorectal cancer risk in the NIH-AARP cohort study. Cancer Causes Control. 2019 Oct;30(10):1127–35.

74. Nishi M, Yoshida K, Hirata K, Miyake H. Eating habits and colorectal cancer. Oncol Rep [Internet]. 1997 Sep 1 [cited 2022 Mar 18]; Available from: http://www.spandidos-publications.com/10.3892/or.4.5.995

75. Santarelli R, Pierre F, Corpet D. Processed Meat and Colorectal Cancer: A Review of Epidemiologic and Experimental Evidence. Nutr Cancer. 2008 Mar;60(2):131–44.

76. Hur J, Otegbeye E, Joh H-K, Nimptsch K, Ng K, Ogino S, et al. Sugar-sweetened beverage intake in adulthood and adolescence and risk of early-onset colorectal cancer among women. Gut. 2021 Dec;70(12):2330–6.

77. Rosato V, Bosetti C, Levi F, Polesel J, Zucchetto A, Negri E, et al. Risk factors for young-onset colorectal cancer. Cancer Causes Control. 2013 Feb;24(2):335–41.

78. Grivas S, Nyhammar T, Olsson K, Jägerstad M. Isolation and identification of the food mutagens IQ and MeIQx from a heated model system of creatinine, glycine and fructose. Food Chem. 1986 Jan;20(2):127–36.

79. Liu P-H, Wu K, Ng K, Zauber AG, Nguyen LH, Song M, et al. Association of Obesity With Risk of Early-Onset Colorectal Cancer Among Women. JAMA Oncol. 2019 Jan 1;5(1):37.

80. Kim JY, Jung YS, Park JH, Kim HJ, Cho YK, Sohn CI, et al. Different risk factors for advanced colorectal neoplasm in young adults. World J Gastroenterol. 2016;22(13):3611.

81. Modzelewska P, Chludzińska S, Lewko J, Reszeć J. The influence of leptin on the process of carcinogenesis. Contemp Oncol Poznan Pol. 2019;23(2):63–8.

82. Endo H, Hosono K, Uchiyama T, Sakai E, Sugiyama M, Takahashi H, et al. Leptin acts as a growth factor for colorectal tumours at stages subsequent to tumour initiation in murine colon carcinogenesis. Gut. 2011 Oct 1;60(10):1363–71.

83. Koda M, Sulkowska M, Kanczuga-Koda L, Surmacz E, Sulkowski S. Overexpression of the obesity hormone leptin in human colorectal cancer. J Clin Pathol. 2007 Aug;60(8):902–6.

84. Singh P, Rubin N. Insulinlike growth factors and binding proteins in colon cancer. Gastroenterology. 1993 Oct;105(4):1218–37.

85. Vigneri PG, Tirrò E, Pennisi MS, Massimino M, Stella S, Romano C, et al. The Insulin/IGF System in Colorectal Cancer Development and Resistance to Therapy. Front Oncol. 2015;5:230.

86. Chen J, Katsifis A, Hu C, Huang X-F. Insulin Decreases Therapeutic Efficacy in Colon Cancer Cell Line HT29 Via the Activation of the PI3K/Akt Pathway. Curr Drug Discov Technol. 2011 Jun 1;8(2):119–25.

87. Rasic I, Rebic V, Rasic A, Aksamija G, Radovic S. The Association of Simultaneous Increase in Interleukin-6, C Reactive Protein, and Matrix Metalloproteinase-9 Serum Levels with Increasing Stages of Colorectal Cancer. J Oncol. 2018 Jul 30;2018:1–7.

88. Himbert C, Ose J, Lin T, Warby CA, Gigic B, Steindorf K, et al. Inflammation- and angiogenesis-related biomarkers are correlated with cancer-related fatigue in colorectal cancer patients: Results from the ColoCare Study. Eur J Cancer Care (Engl). 2019 Jul;28(4):e13055.

89. Riondino S, Roselli M, Palmirotta R, Della-Morte D, Ferroni P, Guadagni F. Obesity and colorectal cancer: role of adipokines in tumor initiation and progression. World J Gastroenterol. 2014 May 14;20(18):5177–90.

90. Pikarsky E, Porat RM, Stein I, Abramovitch R, Amit S, Kasem S, et al. NF-κB functions as a tumour promoter in inflammation-associated cancer. Nature. 2004 Sep;431(7007):461–6.

91. Ward ZJ, Long MW, Resch SC, Giles CM, Cradock AL, Gortmaker SL. Simulation of Growth Trajectories of Childhood Obesity into Adulthood. N Engl J Med. 2017 Nov 30;377(22):2145–53.

92. Ward ZJ, Bleich SN, Cradock AL, Barrett JL, Giles CM, Flax C, et al. Projected U.S. State-Level Prevalence of Adult Obesity and Severe Obesity. N Engl J Med. 2019 Dec 19;381(25):2440–50.

93. de Moura e Dias M, dos Reis SA, da Conceição LL, Sediyama CMN de O, Pereira SS, de Oliveira LL, et al. Diet-induced obesity in animal models: points to consider and influence on metabolic markers. Diabetol Metab Syndr. 2021 Dec;13(1):32.

94. Malik VS, Hu FB. The role of sugar-sweetened beverages in the global epidemics of obesity and chronic diseases. Nat Rev Endocrinol. 2022 Apr;18(4):205–18.

95. Kanarek RB, Orthen-Gambill N. Differential Effects of Sucrose, Fructose and Glucose on Carbohydrate-Induced Obesity in Rats. J Nutr. 1982 Aug 1;112(8):1546–54.

96. Sclafani A. Carbohydrate-induced hyperphagia and obesity in the rat: Effects of saccharide type, form, and taste. Neurosci Biobehav Rev. 1987 Jun;11(2):155–62.

97. Marks-Kaufman R, Hamm MW, Barbato GF. The effects of dietary sucrose on opiate receptor binding in genetically obese (ob/ob) and lean mice. J Am Coll Nutr. 1989 Feb;8(1):9–14.

98. Cao D, Lu H, Lewis TL, Li L. Intake of Sucrose-sweetened Water Induces Insulin Resistance and Exacerbates Memory Deficits and Amyloidosis in a Transgenic Mouse Model of Alzheimer Disease. J Biol Chem. 2007 Dec;282(50):36275–82.

99. Marques C, Meireles M, Norberto S, Leite J, Freitas J, Pestana D, et al. High-fat diet-induced obesity Rat model: a comparison between Wistar and Sprague-Dawley Rat. Adipocyte. 2016 Feb 3;5(1):11–21.

100. Jürgens H, Haass W, Castañeda TR, Schürmann A, Koebnick C, Dombrowski F, et al. Consuming Fructose-sweetened Beverages Increases Body Adiposity in Mice. Obes Res. 2005 Jul;13(7):1146–56.

101. Tillman EJ, Morgan DA, Rahmouni K, Swoap SJ. Three Months of High-Fructose Feeding Fails to Induce Excessive Weight Gain or Leptin Resistance in Mice. Chowen JA, editor. PLoS ONE. 2014 Sep 11;9(9):e107206.

102. Togo J, Hu S, Li M, Niu C, Speakman JR. Impact of dietary sucrose on adiposity and glucose homeostasis in C57BL/6J mice depends on mode of ingestion: liquid or solid. Mol Metab. 2019 Sep;27:22–32.

103. Matias A, Estevam W, Coelho P, Haese D, Kobi J, Lima-Leopoldo A, et al. Differential Effects of High Sugar, High Lard or a Combination of Both on Nutritional, Hormonal and Cardiovascular Metabolic Profiles of Rodents. Nutrients. 2018 Aug 11;10(8):1071.

104. Bell RR, Spencer MJ, Sherriff JL. Voluntary Exercise and Monounsaturated Canola Oil Reduce Fat Gain in Mice Fed Diets High in Fat. J Nutr. 1997 Oct 1;127(10):2006–10.

105. Buettner R, Schölmerich J, Bollheimer LC. High-fat Diets: Modeling the Metabolic Disorders of Human Obesity in Rodents*. Obesity. 2007 Apr;15(4):798–808.

106. Lee Y-Y, Tang T-K, Phuah E-T, Karim NAA, Alitheen NBM, Tan C-P, et al. Structural difference of palm based Medium- and Long-Chain Triacylglycerol (MLCT) further reduces body fat accumulation in DIO C57BL/6J mice when consumed in low fat diet for a mid-term period. Food Res Int. 2018 Jan;103:200–7.

107. Higa TS, Spinola AV, Fonseca-Alaniz MH, Evangelista FSA. Comparison between cafeteria and high-fat diets in the induction of metabolic dysfunction in mice. Int J Physiol Pathophysiol Pharmacol. 2014;6(1):47–54.

108. Bortolin RC, Vargas AR, Gasparotto J, Chaves PR, Schnorr CE, Martinello KB, et al. A new animal diet based on human Western diet is a robust diet-induced obesity model: comparison to high-fat and cafeteria diets in term of metabolic and gut microbiota disruption. Int J Obes. 2018 Mar;42(3):525–34.

109. Doerner SK, Reis ES, Leung ES, Ko JS, Heaney JD, Berger NA, et al. High-Fat Diet-Induced Complement Activation Mediates Intestinal Inflammation and Neoplasia, Independent of Obesity. Mol Cancer Res. 2016 Oct;14(10):953–65.

110. Shan Z, Rehm CD, Rogers G, Ruan M, Wang DD, Hu FB, et al. Trends in Dietary Carbohydrate, Protein, and Fat Intake and Diet Quality Among US Adults, 1999-2016. JAMA. 2019 Sep 24;322(12):1178–87.

111. Blancas-Velazquez A, la Fleur SE, Mendoza J. Effects of a free-choice high-fat high-sugar diet on brain PER2 and BMAL1 protein expression in mice. Appetite. 2017 Oct;117:263–9.

112. Savetsky IL, Albano NJ, Cuzzone DA, Gardenier JC, Torrisi JS, García Nores GD, et al. Lymphatic Function Regulates Contact Hypersensitivity Dermatitis in Obesity. J Invest Dermatol. 2015 Nov;135(11):2742–52.

113. Schemmel R, Mickelsen O, Gill JL. Dietary Obesity in Rats: Body Weight and Body Fat Accretion in Seven Strains of Rats. J Nutr. 1970 Sep 1;100(9):1041–8.

114. Montgomery MK, Hallahan NL, Brown SH, Liu M, Mitchell TW, Cooney GJ, et al. Mouse strain-dependent variation in obesity and glucose homeostasis in response to high-fat feeding. Diabetologia. 2013 May;56(5):1129–39.

115. Alexander J, Chang GQ, Dourmashkin JT, Leibowitz SF. Distinct phenotypes of obesity-prone AKR/J, DBA2J and C57BL/6J mice compared to control strains. Int J Obes. 2006 Jan;30(1):50–9.

116. Rossmeisl M, Rim JS, Koza RA, Kozak LP. Variation in Type 2 Diabetes-Related Traits in Mouse Strains Susceptible to Diet-Induced Obesity. Diabetes. 2003 Aug 1;52(8):1958–66.

117. Surwit RS, Feinglos MN, Rodin J, Sutherland A, Petro AE, Opara EC, et al. Differential effects of fat and sucrose on the development of obesity and diabetes in C57BL/6J and mice. Metabolism. 1995 May;44(5):645–51.

118. Glendinning JI, Breinager L, Kyrillou E, Lacuna K, Rocha R, Sclafani A. Differential effects of sucrose and fructose on dietary obesity in four mouse strains. Physiol Behav. 2010 Oct;101(3):331–43.

119. Yang Z-H, Miyahara H, Takeo J, Katayama M. Diet high in fat and sucrose induces rapid onset of obesity-related metabolic syndrome partly through rapid response of genes involved in lipogenesis, insulin signalling and inflammation in mice. Diabetol Metab Syndr. 2012 Dec;4(1):32.

120. Salinero AE, Anderson BM, Zuloaga KL. Sex differences in the metabolic effects of diet-induced obesity vary by age of onset. Int J Obes. 2018 May;42(5):1088–91.

121. Krishna KB, Stefanovic-Racic M, Dedousis N, Sipula I, O'Doherty RM. Similar degrees of obesity induced by diet or aging cause strikingly different immunologic and metabolic outcomes. Physiol Rep. 2016 Mar;4(6):e12708.

122. Coleman DL. Obese and diabetes: Two mutant genes causing diabetes-obesity syndromes in mice. Diabetologia. 1978 Mar;14(3):141–8.

123. Izquierdo AG, Crujeiras AB, Casanueva FF, Carreira MC. Leptin, Obesity, and Leptin Resistance: Where Are We 25 Years Later? Nutrients. 2019 Nov 8;11(11):2704.

124. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. Nature. 1994 Dec;372(6505):425–32.

125. Considine RV, Sinha MK, Heiman ML, Kriauciunas A, Stephens TW, Nyce MR, et al. Serum Immunoreactive-Leptin Concentrations in Normal-Weight and Obese Humans. N Engl J Med. 1996 Feb;334(5):292–5.

126. Pérusse L, Bouchard C. Gene-diet interactions in obesity. Am J Clin Nutr. 2000 Nov 1;72(5):1285s–90s.

127. Heianza Y, Qi L. Gene-Diet Interaction and Precision Nutrition in Obesity. Int J Mol Sci. 2017 Apr 7;18(4):787.

128. Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? The Lancet. 2001 Feb;357(9255):539–45.

129. Hoebe K, Janssen E, Beutler B. The interface between innate and adaptive immunity. Nat Immunol. 2004 Oct;5(10):971–4.

130. Gajewski TF, Schreiber H, Fu Y-X. Innate and adaptive immune cells in the tumor microenvironment. Nat Immunol. 2013 Oct;14(10):1014–22.

131. Medzhitov R. Origin and physiological roles of inflammation. Nature. 2008 Jul;454(7203):428–35.

132. Flier JS, Underhill LH, Dvorak HF. Tumors: Wounds That Do Not Heal. N Engl J Med. 1986 Dec 25;315(26):1650–9.

133. Chang RB, Beatty GL. The interplay between innate and adaptive immunity in cancer shapes the productivity of cancer immunosurveillance. J Leukoc Biol. 2020 Jul;108(1):363–76.

134. Wang DJ, Ratnam NM, Byrd JC, Guttridge DC. NF-κB functions in tumor initiation by suppressing the surveillance of both innate and adaptive immune cells. Cell Rep. 2014 Oct 9;9(1):90–103.

135. Coussens LM, Werb Z. Inflammation and cancer. Nature. 2002 Dec;420(6917):860–7.

136. Kershaw EE, Flier JS. Adipose Tissue as an Endocrine Organ. J Clin Endocrinol Metab. 2004 Jun;89(6):2548–56.

137. Pou KM, Massaro JM, Hoffmann U, Vasan RS, Maurovich-Horvat P, Larson MG, et al. Visceral and Subcutaneous Adipose Tissue Volumes Are Cross-Sectionally Related to Markers of Inflammation and Oxidative Stress: The Framingham Heart Study. Circulation. 2007 Sep 11;116(11):1234–41.

138. Schlecht I, Fischer B, Behrens G, Leitzmann MF. Relations of Visceral and Abdominal Subcutaneous Adipose Tissue, Body Mass Index, and Waist Circumference to Serum Concentrations of Parameters of Chronic Inflammation. Obes Facts. 2016;9(3):144–57.

139. Wisse BE. The Inflammatory Syndrome: The Role of Adipose Tissue Cytokines in Metabolic Disorders Linked to Obesity. J Am Soc Nephrol. 2004 Nov 1;15(11):2792–800.

140. Cancello R, Henegar C, Viguerie N, Taleb S, Poitou C, Rouault C, et al. Reduction of Macrophage Infiltration and Chemoattractant Gene Expression Changes in White Adipose Tissue of Morbidly Obese Subjects After Surgery-Induced Weight Loss. Diabetes. 2005 Aug 1;54(8):2277–86.

141. Curat CA, Wegner V, Sengenès C, Miranville A, Tonus C, Busse R, et al. Macrophages in human visceral adipose tissue: increased accumulation in obesity and a source of resistin and visfatin. Diabetologia. 2006 Apr;49(4):744–7.

142. Fried SK, Bunkin DA, Greenberg AS. Omental and Subcutaneous Adipose Tissues of Obese Subjects Release Interleukin-6: Depot Difference and Regulation by Glucocorticoid [1]. J Clin Endocrinol Metab. 1998 Mar;83(3):847–50.

143. Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science. 1993 Jan 1;259(5091):87–91.

144. Cinti S, Mitchell G, Barbatelli G, Murano I, Ceresi E, Faloia E, et al. Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. J Lipid Res. 2005 Nov;46(11):2347–55.

145. Altintas MM, Azad A, Nayer B, Contreras G, Zaias J, Faul C, et al. Mast cells, macrophages, and crown-like structures distinguish subcutaneous from visceral fat in mice. J Lipid Res. 2011 Mar;52(3):480–8.

146. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW. Obesity is associated with macrophage accumulation in adipose tissue. J Clin Invest. 2003 Dec 15;112(12):1796–808.

147. Xia L, Tan S, Zhou Y, Lin J, Wang H, Oyang L, et al. Role of the NFκB-signaling pathway in cancer. OncoTargets Ther. 2018;11:2063–73.

148. Yu H, Pardoll D, Jove R. STATs in cancer inflammation and immunity: a leading role for STAT3. Nat Rev Cancer. 2009 Nov;9(11):798–809.

149. Pendyala S, Neff LM, Suárez-Fariñas M, Holt PR. Diet-induced weight loss reduces colorectal inflammation: implications for colorectal carcinogenesis. Am J Clin Nutr. 2011 Feb;93(2):234–42.

150. Pfalzer AC, Leung K, Crott JW, Kim SJ, Tai AK, Parnell LD, et al. Incremental Elevations in TNFα and IL6 in the Human Colon and Procancerous Changes in the Mucosal Transcriptome Accompany Adiposity. Cancer Epidemiol Biomark Prev Publ Am Assoc Cancer Res Cosponsored Am Soc Prev Oncol. 2018 Dec;27(12):1416–23.

151. Gulhane M, Murray L, Lourie R, Tong H, Sheng YH, Wang R, et al. High Fat Diets Induce Colonic Epithelial Cell Stress and Inflammation that is Reversed by IL-22. Sci Rep. 2016 Jul;6(1):28990.

152. Luck H, Tsai S, Chung J, Clemente-Casares X, Ghazarian M, Revelo XS, et al. Regulation of Obesity-Related Insulin Resistance with Gut Anti-inflammatory Agents. Cell Metab. 2015 Apr;21(4):527–42.

153. Alard J, Lehrter V, Rhimi M, Mangin I, Peucelle V, Abraham A-L, et al. Beneficial metabolic effects of selected probiotics on diet-induced obesity and insulin resistance in mice are associated with improvement of dysbiotic gut microbiota: Probiotics, obesity and gut microbiota. Environ Microbiol. 2016 May;18(5):1484–97.

154. Castaner O, Goday A, Park Y-M, Lee S-H, Magkos F, Shiow S-ATE, et al. The Gut Microbiome Profile in Obesity: A Systematic Review. Int J Endocrinol. 2018;2018:1–9.

155. Garrett WS. The gut microbiota and colon cancer. Science. 2019 Jun 21;364(6446):1133–5.

156. Garrett WS. Immune recognition of microbial metabolites. Nat Rev Immunol. 2020 Feb;20(2):91–2.

157. Sonnenburg JL, Bäckhed F. Diet–microbiota interactions as moderators of human metabolism. Nature. 2016 Jul 7;535(7610):56–64.

158. Hill MJ. Intestinal flora and endogenous vitamin synthesis: Eur J Cancer Prev. 1997 Mar;6:S43–5.

159. Bäckhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI. Host-Bacterial Mutualism in the Human Intestine. Science. 2005 Mar 25;307(5717):1915–20.

160. Molinero N, Ruiz L, Sánchez B, Margolles A, Delgado S. Intestinal Bacteria Interplay With Bile and Cholesterol Metabolism: Implications on Host Physiology. Front Physiol. 2019 Mar 14;10:185.

161. MetaHIT Consortium, Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature. 2010 Mar;464(7285):59–65.

162. Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende DR, et al. Enterotypes of the human gut microbiome. Nature. 2011 May 12;473(7346):174–80.

163. van Leeuwenhoek A. Letter66.In:Letter of Leeuwenhoek to Robert Hooke [Internet]. Available from: https://www.dbnl.org/tekst/leeu027alle003_001/leeu027alle003_001_0025.php%23b00661681; https://www.dbnl.org/tekst/leeu027alle003_001/leeu027alle003_001_0025.php#b0066

164. Escherich T. Die Darmbakterien des Neugeborenen und Säuglings. FortschderMed. 1885;(3):515–22, 547–54.

165. Escherich T. Die Darmbakterien des Säuglings und ihre Beziehungen zur Physiologie der Berdauung. 1886;

166. Tissier H. La réaction cromophile d'Escherich et le bacterium coli. Comp rend Soc de Biol. 1899;943–5.

167. Tissier H. Étude d'une varieté d'infection intestinale chez le nourrisson. Annales Institut Pasteur. 1905;19:274–316.

168. Tissier H. Traitement des infections intestinales par la m´ethode de transformation de la flore bacteri´ene de l'intestin. Comptes rendus des séances de la Société de Biologie et de ses filiales. 51(T1):359.

169. Metchnikov I. Les microbes intestinaux Revue. Bulletin de l'Institut Pasteur. 1903;

170. Farré-Maduell E, Casals-Pascual C. The origins of gut microbiome research in Europe: From Escherich to Nissle. Hum Microbiome J. 2019 Dec;14:100065.

171. Bäckhed F, Ding H, Wang T, Hooper LV, Koh GY, Nagy A, et al. The gut microbiota as an environmental factor that regulates fat storage. Proc Natl Acad Sci U S A. 2004 Nov 2;101(44):15718–23.

172. Bäckhed F, Manchester JK, Semenkovich CF, Gordon JI. Mechanisms underlying the resistance to diet-induced obesity in germ-free mice. Proc Natl Acad Sci U S A. 2007 Jan 16;104(3):979–84.

173. Human Microbiome Project Consortium. Structure, function and diversity of the healthy human microbiome. Nature. 2012 Jun 13;486(7402):207–14.

174. Nelson KE, editor. Metagenomics of the human body. New York: Springer; 2011. 351 p.

175. Weir TL, Manter DK, Sheflin AM, Barnett BA, Heuberger AL, Ryan EP. Stool Microbiome and Metabolome Differences between Colorectal Cancer Patients and Healthy Adults. White BA, editor. PLoS ONE. 2013 Aug 6;8(8):e70803.

176. Sobhani I, Tap J, Roudot-Thoraval F, Roperch JP, Letulle S, Langella P, et al. Microbial Dysbiosis in Colorectal Cancer (CRC) Patients. Pied S, editor. PLoS ONE. 2011 Jan 27;6(1):e16393.

177. Li L, Li X, Zhong W, Yang M, Xu M, Sun Y, et al. Gut microbiota from colorectal cancer patients enhances the progression of intestinal adenoma in Apcmin/+ mice. EBioMedicine. 2019 Oct;48:301–15.

178. Wu N, Yang X, Zhang R, Li J, Xiao X, Hu Y, et al. Dysbiosis Signature of Fecal Microbiota in Colorectal Cancer Patients. Microb Ecol. 2013 Aug;66(2):462–70.

179. Pastille E, Faßnacht T, Adamczyk A, Ngo Thi Phuong N, Buer J, Westendorf AM. Inhibition of TLR4 Signaling Impedes Tumor Growth in Colitis-Associated Colon Cancer. Front Immunol. 2021 May 7;12:669747.

180. Cani PD, Bibiloni R, Knauf C, Waget A, Neyrinck AM, Delzenne NM, et al. Changes in Gut Microbiota Control Metabolic Endotoxemia-Induced Inflammation in High-Fat Diet–Induced Obesity and Diabetes in Mice. Diabetes. 2008 Jun 1;57(6):1470–81.

181. Ke W, Bonilla-Rosso G, Engel P, Wang P, Chen F, Hu X. Suppression of High-Fat Diet-Induced Obesity by Platycodon Grandiflorus in Mice Is Linked to Changes in the Gut Microbiota. J Nutr. 2020 Sep 1;150(9):2364–74.

182. Burrello C, Garavaglia F, Cribiù FM, Ercoli G, Lopez G, Troisi J, et al. Therapeutic faecal microbiota transplantation controls intestinal inflammation through IL10 secretion by immune cells. Nat Commun. 2018 Dec;9(1):5184.

183. Burrello C, Giuffrè MR, Macandog AD, Diaz-Basabe A, Cribiù FM, Lopez G, et al. Fecal Microbiota Transplantation Controls Murine Chronic Intestinal Inflammation by Modulating Immune Cell Functions and Gut Microbiota Composition. Cells. 2019 May 28;8(6):517.

184. Zackular JP, Baxter NT, Chen GY, Schloss PD. Manipulation of the Gut Microbiota Reveals Role in Colon Tumorigenesis. mSphere. 2016 Feb;1(1):e00001-15.

185. Zackular JP, Baxter NT, Iverson KD, Sadler WD, Petrosino JF, Chen GY, et al. The gut microbiome modulates colon tumorigenesis. mBio. 2013 Nov 5;4(6):e00692-00613.

186. de La Serre CB, Ellis CL, Lee J, Hartman AL, Rutledge JC, Raybould HE. Propensity to high-fat diet-induced obesity in rats is associated with changes in the gut microbiota and gut inflammation. Am J Physiol-Gastrointest Liver Physiol. 2010 Aug;299(2):G440–8.

187. Nguyen TLA, Vieira-Silva S, Liston A, Raes J. How informative is the mouse for human gut microbiota research? Dis Model Mech. 2015 Jan 1;8(1):1–16.

188. Krych L, Hansen CHF, Hansen AK, van den Berg FWJ, Nielsen DS. Quantitatively Different, yet Qualitatively Alike: A Meta-Analysis of the Mouse Core Gut Microbiome with a View towards the Human Gut Microbiome. Bereswill S, editor. PLoS ONE. 2013 May 1;8(5):e62578.

189. Xiao L, Feng Q, Liang S, Sonne SB, Xia Z, Qiu X, et al. A catalog of the mouse gut metagenome. Nat Biotechnol. 2015 Oct;33(10):1103–8.

190. Hugenholtz F, de Vos WM. Mouse models for human intestinal microbiota research: a critical evaluation. Cell Mol Life Sci. 2018 Jan;75(1):149–60.

191. Depommier C, Everard A, Druart C, Plovier H, Van Hul M, Vieira-Silva S, et al. Supplementation with Akkermansia muciniphila in overweight and obese human volunteers: a proof-of-concept exploratory study. Nat Med. 2019 Jul;25(7):1096–103.

192. Plovier H, Everard A, Druart C, Depommier C, Van Hul M, Geurts L, et al. A purified membrane protein from Akkermansia muciniphila or the pasteurized bacterium improves metabolism in obese and diabetic mice. Nat Med. 2017 Jan;23(1):107–13.

193. Pleasants JR. REARING GERMFREE CESAREAN-BORN RATS, MICE, AND RABBITS THROUGH WEANING*. Ann N Y Acad Sci. 2006 Dec 15;78(1):116–26.

194. Round JL, Mazmanian SK. The gut microbiota shapes intestinal immune responses during health and disease. Nat Rev Immunol. 2009 May;9(5):313–23.

195. Lundberg R, Toft MF, August B, Hansen AK, Hansen CHF. Antibiotic-treated versus germ-free rodents for microbiota transplantation studies. Gut Microbes. 2016 Jan 2;7(1):68–74.

196. Tirelle P, Breton J, Riou G, Déchelotte P, Coëffier M, Ribet D. Comparison of different modes of antibiotic delivery on gut microbiota depletion efficiency and body composition in mouse. BMC Microbiol. 2020 Nov 11;20(1):340.

197. Sarrabayrouse G, Landolfi S, Pozuelo M, Willamil J, Varela E, Clark A, et al. Mucosal microbial load in Crohn's disease: A potential predictor of response to faecal microbiota transplantation. EBioMedicine. 2020 Jan;51:102611.

198. Kennedy EA, King KY, Baldridge MT. Mouse Microbiota Models: Comparing Germ-Free Mice and Antibiotics Treatment as Tools for Modifying Gut Bacteria. Front Physiol. 2018 Oct 31;9:1534.

199. Hill DA, Hoffmann C, Abt MC, Du Y, Kobuley D, Kirn TJ, et al. Metagenomic analyses reveal antibiotic-induced temporal and spatial changes in intestinal microbiota with associated alterations in immune cell homeostasis. Mucosal Immunol. 2010 Mar;3(2):148–58.

200. Bokoliya SC, Dorsett Y, Panier H, Zhou Y. Procedures for Fecal Microbiota Transplantation in Murine Microbiome Studies. Front Cell Infect Microbiol. 2021 Sep 21;11:711055.

201. Zhou J, Zhou Z, Ji P, Ma M, Guo J, Jiang S. Effect of fecal microbiota transplantation on experimental colitis in mice. Exp Ther Med [Internet]. 2019 Feb 13 [cited 2022 Jan 15]; Available from: http://www.spandidos-publications.com/10.3892/etm.2019.7263

202. Wrzosek L, Ciocan D, Borentain P, Spatz M, Puchois V, Hugot C, et al. Transplantation of human microbiota into conventional mice durably reshapes the gut microbiota. Sci Rep. 2018 Dec;8(1):6854.

203. Zhang L, Bahl MI, Roager HM, Fonvig CE, Hellgren LI, Frandsen HL, et al. Environmental spread of microbes impacts the development of metabolic phenotypes in mice transplanted with microbial communities from humans. ISME J. 2017 Mar;11(3):676–90.

204. Hugenholtz P, Pace N. Identifying microbial diversity in the natural environment: A molecular phylogenetic approach. Trends Biotechnol. 1996 Jun;14(6):190–7.

205. Hunyady B, Mezey E, Palkovits M. Gastrointestinal immunology: cell types in the lamina propria--a morphological review. Acta Physiol Hung. 2000;87(4):305–28.

206. van der Wath RC, Gardiner BS, Burgess AW, Smith DW. Cell Organisation in the Colonic Crypt: A Theoretical Comparison of the Pedigree and Niche Concepts. Chan C, editor. PLoS ONE. 2013 Sep 12;8(9):e73204.

207. Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R. Recognition of Commensal Microflora by Toll-Like Receptors Is Required for Intestinal Homeostasis. Cell. 2004 Jul;118(2):229–41.

208. Chu H, Mazmanian SK. Innate immune recognition of the microbiota promotes host-microbial symbiosis. Nat Immunol. 2013 Jul;14(7):668–75.

209. Mazmanian SK, Liu CH, Tzianabos AO, Kasper DL. An Immunomodulatory Molecule of Symbiotic Bacteria Directs Maturation of the Host Immune System. Cell. 2005 Jul;122(1):107–18.

210. Round JL, Mazmanian SK. Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. Proc Natl Acad Sci. 2010 Jul 6;107(27):12204–9.

211. Isidro RA, Appleyard CB. Colonic macrophage polarization in homeostasis, inflammation, and cancer. Am J Physiol-Gastrointest Liver Physiol. 2016 Jul 1;311(1):G59–73.

212. Mortha A, Chudnovskiy A, Hashimoto D, Bogunovic M, Spencer SP, Belkaid Y, et al. Microbiota-Dependent Crosstalk Between Macrophages and ILC3 Promotes Intestinal Homeostasis. Science. 2014 Mar 28;343(6178):1249288.

213. Strati F, Pujolassos M, Burrello C, Giuffrè MR, Lattanzi G, Caprioli F, et al. Antibiotic-associated dysbiosis affects the ability of the gut microbiota to control intestinal inflammation upon fecal microbiota transplantation in experimental colitis models. Microbiome. 2021 Dec;9(1):39.

214. Kim MH, Kang SG, Park JH, Yanagisawa M, Kim CH. Short-Chain Fatty Acids Activate GPR41 and GPR43 on Intestinal Epithelial Cells to Promote Inflammatory Responses in Mice. Gastroenterology. 2013 Aug;145(2):396-406.e10.

215. Kang E, Zhou G, Yousefi M, Cayrol R, Xia J, Gruenheid S. Loss of disease tolerance during Citrobacter rodentium infection is associated with impaired epithelial differentiation and hyperactivation of T cell responses. Sci Rep. 2018 Dec;8(1):847.

216. Litvak Y, Byndloss MX, Bäumler AJ. Colonocyte metabolism shapes the gut microbiota. Science. 2018 Nov 30;362(6418):eaat9076.

217. Cevallos SA, Lee J-Y, Velazquez EM, Foegeding NJ, Shelton CD, Tiffany CR, et al. 5-Aminosalicylic Acid Ameliorates Colitis and Checks Dysbiotic Escherichia coli Expansion by Activating PPAR-γ Signaling in the Intestinal Epithelium. Ehrt S, editor. mBio. 2021 Feb 23;12(1):e03227-20.

218. Shi N, Li N, Duan X, Niu H. Interaction between the gut microbiome and mucosal immune system. Mil Med Res. 2017;4:14.

219. Nepelska M, de Wouters T, Jacouton E, Béguet-Crespel F, Lapaque N, Doré J, et al. Commensal gut bacteria modulate phosphorylation-dependent PPARγ transcriptional activity in human intestinal epithelial cells. Sci Rep. 2017 Mar;7(1):43199.

220. Byndloss MX, Olsan EE, Rivera-Chávez F, Tiffany CR, Cevallos SA, Lokken KL, et al. Microbiota-activated PPAR-γ signaling inhibits dysbiotic Enterobacteriaceae expansion. Science. 2017 Aug 11;357(6351):570–5.

221. Ahmadian M, Suh JM, Hah N, Liddle C, Atkins AR, Downes M, et al. PPARγ signaling and metabolism: the good, the bad and the future. Nat Med. 2013 May;19(5):557–66.

222. Shin N-R, Whon TW, Bae J-W. Proteobacteria: microbial signature of dysbiosis in gut microbiota. Trends Biotechnol. 2015 Sep;33(9):496–503.

223. Furuta GT, Turner JR, Taylor CT, Hershberg RM, Comerford K, Narravula S, et al. Hypoxia-Inducible Factor 1–Dependent Induction of Intestinal Trefoil Factor Protects Barrier Function during Hypoxia. J Exp Med. 2001 May 7;193(9):1027–34.

224. Kelly CJ, Glover LE, Campbell EL, Kominsky DJ, Ehrentraut SF, Bowers BE, et al. Fundamental role for HIF-1α in constitutive expression of human β defensin-1. Mucosal Immunol. 2013 Nov;6(6):1110–8.

225. Louis NA, Hamilton KE, Canny G, Shekels LL, Ho SB, Colgan SP. Selective induction of mucin-3 by hypoxia in intestinal epithelia. J Cell Biochem. 2006 Dec 15;99(6):1616–27.

226. Sarraf P, Mueller E, Jones D, King FJ, DeAngelo DJ, Partridge JB, et al. Differentiation and reversal of malignant changes in colon cancer through PPARγ. Nat Med. 1998 Sep;4(9):1046–52.

227. Zhao J, Zhao R, Cheng L, Yang J, Zhu L. Peroxisome proliferator-activated receptor gamma activation promotes intestinal barrier function by improving mucus and tight junctions in a mouse colitis model. Dig Liver Dis. 2018 Nov;50(11):1195–204.

228. Fajas L, Auboeuf D, Raspé E, Schoonjans K, Lefebvre A-M, Saladin R, et al. The Organization, Promoter Analysis, and Expression of the Human PPARγ Gene. J Biol Chem. 1997 Jul;272(30):18779–89.

229. Lefebvre M, Paulweber B, Fajas L, Woods J, McCrary C, Colombel J, et al. Peroxisome proliferator-activated receptor gamma is induced during differentiation of colon epithelium cells. J Endocrinol. 1999 Sep 1;162(3):331–40.

230. Su W, Bush CR, Necela BM, Calcagno SR, Murray NR, Fields AP, et al. Differential expression, distribution, and function of PPAR-γ in the proximal and distal colon. Physiol Genomics. 2007 Aug;30(3):342–53.

231. Vellend M. Conceptual Synthesis in Community Ecology. Q Rev Biol. 2010 Jun;85(2):183–206.

232. Stegen JC, Lin X, Konopka AE, Fredrickson JK. Stochastic and deterministic assembly processes in subsurface microbial communities. ISME J. 2012 Sep;6(9):1653–64.

233. Sung J, Kim S, Cabatbat JJT, Jang S, Jin Y-S, Jung GY, et al. Global metabolic interaction network of the human gut microbiota for context-specific community-scale analysis. Nat Commun. 2017 Aug;8(1):15393.

234. Smith NW, Shorten PR, Altermann E, Roy NC, McNabb WC. The Classification and Evolution of Bacterial Cross-Feeding. Front Ecol Evol. 2019 May 14;7:153.

235. Brennan CA, Garrett WS. Gut Microbiota, Inflammation, and Colorectal Cancer. Annu Rev Microbiol. 2016 Sep 8;70(1):395–411.

236. Cheng Y, Ling Z, Li L. The Intestinal Microbiota and Colorectal Cancer. Front Immunol. 2020 Nov 30;11:615056.

237. Jobin C. Colorectal Cancer: Looking for Answers in the Microbiota. Cancer Discov. 2013 Apr;3(4):384–7.

238. De Filippo C, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, Massart S, et al. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proc Natl Acad Sci. 2010 Aug 17;107(33):14691–6.

239. Walker AW, Ince J, Duncan SH, Webster LM, Holtrop G, Ze X, et al. Dominant and diet-responsive groups of bacteria within the human colonic microbiota. ISME J. 2011 Feb;5(2):220–30.

240. Wu GD, Chen J, Hoffmann C, Bittinger K, Chen Y-Y, Keilbaugh SA, et al. Linking Long-Term Dietary Patterns with Gut Microbial Enterotypes. Science. 2011 Oct 7;334(6052):105–8.

241. David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, et al. Diet rapidly and reproducibly alters the human gut microbiome. Nature. 2014 Jan;505(7484):559–63.

242. Kovatcheva-Datchary P, Nilsson A, Akrami R, Lee YS, De Vadder F, Arora T, et al. Dietary Fiber-Induced Improvement in Glucose Metabolism Is Associated with Increased Abundance of Prevotella. Cell Metab. 2015 Dec;22(6):971–82.

243. Rothschild D, Weissbrod O, Barkan E, Kurilshikov A, Korem T, Zeevi D, et al. Environment dominates over host genetics in shaping human gut microbiota. Nature. 2018 Mar;555(7695):210–5.

244. Carmody RN, Gerber GK, Luevano JM, Gatti DM, Somes L, Svenson KL, et al. Diet dominates host genotype in shaping the murine gut microbiota. Cell Host Microbe. 2015 Jan 14;17(1):72–84.

245. Cotillard A, Kennedy SP, Kong LC, Prifti E, Pons N, Le Chatelier E, et al. Dietary intervention impact on gut microbial gene richness. Nature. 2013 Aug 29;500(7464):585–8.

246. Claesson MJ, Jeffery IB, Conde S, Power SE, O'Connor EM, Cusack S, et al. Gut microbiota composition correlates with diet and health in the elderly. Nature. 2012 Aug;488(7410):178–84.

247. Pace NR, Stahl DA, Lane DJ, Olsen GJ. The Analysis of Natural Microbial Populations by Ribosomal RNA Sequences. In: Marshall KC, editor. Advances in Microbial Ecology [Internet]. Boston, MA: Springer US; 1986 [cited 2021 Dec 20]. p. 1–55. (Advances in Microbial Ecology; vol. 9). Available from: http://link.springer.com/10.1007/978-1-4757-0611-6_1

248. Woese CR, Fox GE, Zablen L, Uchida T, Bonen L, Pechman K, et al. Conservation of primary structure in 16S ribosomal RNA. Nature. 1975 Mar 6;254(5495):83–6.

249. Janda JM, Abbott SL. 16S rRNA Gene Sequencing for Bacterial Identification in the Diagnostic Laboratory: Pluses, Perils, and Pitfalls. J Clin Microbiol. 2007 Sep;45(9):2761–4.

250. Bharti R, Grimm DG. Current challenges and best-practice protocols for microbiome analysis. Brief Bioinform. 2021 Jan 18;22(1):178–93.

251. Van de Peer Y, Chapelle S, De Wachter R. A quantitative map of nucleotide substitution rates in bacterial rRNA. Nucleic Acids Res. 1996 Sep 1;24(17):3381–91.

252. Faith DP. Conservation evaluation and phylogenetic diversity. Biol Conserv. 1992;61(1):1–10.

253. Pielou EC. The measurement of diversity in different types of biological collections. J Theor Biol. 1966 Dec;13:131–44.

254. Shannon CE. A Mathematical Theory of Communication. Bell Syst Tech J. 1948 Jul;27(3):379–423.

255. Bray JR, Curtis JT. An Ordination of the Upland Forest Communities of Southern Wisconsin. Ecol Monogr. 1957 Oct;27(4):325–49.

256. Jaccard P. THE DISTRIBUTION OF THE FLORA IN THE ALPINE ZONE.1. New Phytol. 1912 Feb;11(2):37–50.

257. Lozupone C, Knight R. UniFrac: a New Phylogenetic Method for Comparing Microbial Communities. Appl Environ Microbiol. 2005 Dec;71(12):8228–35.

258. Lozupone CA, Hamady M, Kelley ST, Knight R. Quantitative and Qualitative β Diversity Measures Lead to Different Insights into Factors That Structure Microbial Communities. Appl Environ Microbiol. 2007 Mar;73(5):1576–85.

259. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 2012 Nov 27;41(D1):D590–6.

260. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, et al. Greengenes, a Chimera-Checked 16S rRNA Gene Database and Workbench Compatible with ARB. Appl Environ Microbiol. 2006 Jul;72(7):5069–72.

261. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naïve Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy. Appl Environ Microbiol. 2007 Aug 15;73(16):5261–7.

262. Cole JR, Wang Q, Fish JA, Chai B, McGarrell DM, Sun Y, et al. Ribosomal Database Project: data and tools for high throughput rRNA analysis. Nucleic Acids Res. 2014 Jan;42(D1):D633–42.

263. Turnbaugh PJ, Ridaura VK, Faith JJ, Rey FE, Knight R, Gordon JI. The Effect of Diet on the Human Gut Microbiome: A Metagenomic Analysis in Humanized Gnotobiotic Mice. Sci Transl Med [Internet]. 2009 Nov 11 [cited 2022 Jan 1];1(6). Available from: https://www.science.org/doi/10.1126/scitranslmed.3000322

264. De Angelis M, Ferrocino I, Calabrese FM, De Filippis F, Cavallo N, Siragusa S, et al. Diet influences the functions of the human intestinal microbiome. Sci Rep. 2020 Dec;10(1):4247.

265. Kurokawa K, Itoh T, Kuwahara T, Oshima K, Toh H, Toyoda A, et al. Comparative Metagenomics Revealed Commonly Enriched Gene Sets in Human Gut Microbiomes. DNA Res. 2007;14(4):169–81.

266. Reynolds A, Mann J, Cummings J, Winter N, Mete E, Te Morenga L. Carbohydrate quality and human health: a series of systematic reviews and meta-analyses. The Lancet. 2019 Feb;393(10170):434–45.

267. Tuan J, Chen Y-X. Dietary and Lifestyle Factors Associated with Colorectal Cancer Risk and Interactions with Microbiota: Fiber, Red or Processed Meat and Alcoholic Drinks. Gastrointest Tumors. 2016;3(1):17–24.

268. O'Keefe SJD. Diet, microorganisms and their metabolites, and colon cancer. Nat Rev Gastroenterol Hepatol. 2016 Dec;13(12):691–706.

269. Ou J, DeLany JP, Zhang M, Sharma S, O'Keefe SJD. Association Between Low Colonic Short-Chain Fatty Acids and High Bile Acids in High Colon Cancer Risk Populations. Nutr Cancer. 2012 Jan;64(1):34–40.

270. Bergman EN. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiol Rev. 1990 Apr;70(2):567–90.

271. Sonnenburg ED, Sonnenburg JL. Starving our Microbial Self: The Deleterious Consequences of a Diet Deficient in Microbiota-Accessible Carbohydrates. Cell Metab. 2014 Nov;20(5):779–86.

272. Donohoe DR, Garge N, Zhang X, Sun W, O'Connell TM, Bunger MK, et al. The Microbiome and Butyrate Regulate Energy Metabolism and Autophagy in the Mammalian Colon. Cell Metab. 2011 May;13(5):517–26.

273. Rivière A, Selak M, Lantin D, Leroy F, De Vuyst L. Bifidobacteria and Butyrate-Producing Colon Bacteria: Importance and Strategies for Their Stimulation in the Human Gut. Front Microbiol [Internet]. 2016 Jun 28 [cited 2022 Jan 4];7. Available from: http://journal.frontiersin.org/Article/10.3389/fmicb.2016.00979/abstract

274. Duncan SH, Hold GL, Barcenilla A, Stewart CS, Flint HJ. Roseburia intestinalis sp. nov., a novel saccharolytic, butyrate-producing bacterium from human faeces. Int J Syst Evol Microbiol. 2002 Sep 1;52(5):1615–20.

275. Ferreira-Halder CV, Faria AV de S, Andrade SS. Action and function of Faecalibacterium prausnitzii in health and disease. Best Pract Res Clin Gastroenterol. 2017 Dec;31(6):643–8.

276. Christl SU, Eisner H-D, Dusel G, Kasper H, Scheppach W. Antagonistic effects of sulfide and butyrate on proliferation of colonic mucosa: A potential role for these agents in the pathogenesis of ulcerative colitis. Dig Dis Sci. 1996 Dec;41(12):2477–81.

277. Xi Y, Jing Z, Wei W, Chun Z, Quan Q, Qing Z, et al. Inhibitory effect of sodium butyrate on colorectal cancer cells and construction of the related molecular network. BMC Cancer. 2021 Dec;21(1):127.

278. Thangaraju M, Cresci GA, Liu K, Ananth S, Gnanaprakasam JP, Browning DD, et al. GPR109A Is a G-protein–Coupled Receptor for the Bacterial Fermentation Product Butyrate and Functions as a Tumor Suppressor in Colon. Cancer Res. 2009 Apr 1;69(7):2826–32.

279. Desai MS, Seekatz AM, Koropatkin NM, Kamada N, Hickey CA, Wolter M, et al. A Dietary Fiber-Deprived Gut Microbiota Degrades the Colonic Mucus Barrier and Enhances Pathogen Susceptibility. Cell. 2016 Nov;167(5):1339-1353.e21.

280. Atarashi K, Tanoue T, Oshima K, Suda W, Nagano Y, Nishikawa H, et al. Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. Nature. 2013 Aug 8;500(7461):232–6.

281. Arpaia N, Campbell C, Fan X, Dikiy S, van der Veeken J, deRoos P, et al. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. Nature. 2013 Dec;504(7480):451–5.

282. Gurav A, Sivaprakasam S, Bhutia YD, Boettger T, Singh N, Ganapathy V. Slc5a8, a Na+-coupled high-affinity transporter for short-chain fatty acids, is a conditional tumour suppressor in colon that protects against colitis and colon cancer under low-fibre dietary conditions. Biochem J. 2015 Jul 15;469(2):267–78.

283. Furusawa Y, Obata Y, Fukuda S, Endo TA, Nakato G, Takahashi D, et al. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. Nature. 2013 Dec 19;504(7480):446–50.

284. Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly-Y M, et al. The Microbial Metabolites, Short-Chain Fatty Acids, Regulate Colonic T_{reg} Cell Homeostasis. Science. 2013 Aug 2;341(6145):569–73.

285. Donohoe DR, Holley D, Collins LB, Montgomery SA, Whitmore AC, Hillhouse A, et al. A Gnotobiotic Mouse Model Demonstrates That Dietary Fiber Protects against Colorectal Tumorigenesis in a Microbiota- and Butyrate-Dependent Manner. Cancer Discov. 2014 Dec;4(12):1387–97.

286. Magee EA, Richardson CJ, Hughes R, Cummings JH. Contribution of dietary protein to sulfide production in the large intestine: an in vitro and a controlled feeding study in humans. Am J Clin Nutr. 2000 Dec 1;72(6):1488–94.

287. Peck HD. ENZYMATIC BASIS FOR ASSIMILATORY AND DISSIMILATORY SULFATE REDUCTION. J Bacteriol. 1961 Dec;82(6):933–9.

288. Carbonero F, Benefiel AC, Alizadeh-Ghamsari AH, Gaskins HR. Microbial pathways in colonic sulfur metabolism and links with health and disease. Front Physiol [Internet]. 2012 [cited 2022 Jan 6];3. Available from: http://journal.frontiersin.org/article/10.3389/fphys.2012.00448/abstract

289. Attene-Ramos MS, Wagner ED, Plewa MJ, Gaskins HR. Evidence That Hydrogen Sulfide Is a Genotoxic Agent. Mol Cancer Res. 2006 Jan;4(1):9–14.

290. Attene-Ramos MS, Wagner ED, Gaskins HR, Plewa MJ. Hydrogen Sulfide Induces Direct Radical-Associated DNA Damage. Mol Cancer Res. 2007 May;5(5):455–9.

291. Attene-Ramos MS, Nava GM, Muellner MG, Wagner ED, Plewa MJ, Gaskins HR. DNA damage and toxicogenomic analyses of hydrogen sulfide in human intestinal epithelial FHs 74 Int cells. Environ Mol Mutagen. 2010;NA-NA.

292. Roediger WEW, Duncan A, Kapaniris O, Millard S. Reducing sulfur compounds of the colon impair colonocyte nutrition: Implications for ulcerative colitis. Gastroenterology. 1993 Mar;104(3):802–9.

293. Babidge W, Millard S, Roediger W. Sulfides impair short chain fatty acid beta-oxidation at acyl-CoA dehydrogenase level in colonocytes: implications for ulcerative colitis. Mol Cell Biochem. 1998;181(1/2):117–24.

294. Kostic AD, Gevers D, Pedamallu CS, Michaud M, Duke F, Earl AM, et al. Genomic analysis identifies association of *Fusobacterium* with colorectal carcinoma. Genome Res. 2012 Feb;22(2):292–8.

295. Kostic AD, Chun E, Robertson L, Glickman JN, Gallini CA, Michaud M, et al. Fusobacterium nucleatum Potentiates Intestinal Tumorigenesis and Modulates the Tumor-Immune Microenvironment. Cell Host Microbe. 2013 Aug;14(2):207–15.

296. Bullman S, Pedamallu CS, Sicinska E, Clancy TE, Zhang X, Cai D, et al. Analysis of *Fusobacterium* persistence and antibiotic response in colorectal cancer. Science. 2017 Dec 15;358(6369):1443–8.

297. Yazici C, Wolf PG, Kim H, Cross T-WL, Vermillion K, Carroll T, et al. Race-dependent association of sulfidogenic bacteria with colorectal cancer. Gut. 2017 Nov;66(11):1983–94.

298. Świątecka D, Dominika Ś, Narbad A, Arjan N, Ridgway KP, Karyn RP, et al. The study on the impact of glycated pea proteins on human intestinal bacteria. Int J Food Microbiol. 2011 Jan 31;145(1):267–72.

299. Meddah ATT, Yazourh A, Desmet I, Risbourg B, Verstraete W, Romond MB. The regulatory effects of whey retentate from Bifidobacteria fermented milk on the microbiota of the Simulator of the Human Intestinal Microbial Ecosystem (SHIME). J Appl Microbiol. 2001 Dec;91(6):1110–7.

300. De Weirdt R, Possemiers S, Vermeulen G, Moerdijk-Poortvliet TCW, Boschker HTS, Verstraete W, et al. Human faecal microbiota display variable patterns of glycerol metabolism: Glycerol metabolism in the human colon. FEMS Microbiol Ecol. 2010 Dec;74(3):601–11.

301. Schaefer L, Auchtung TA, Hermans KE, Whitehead D, Borhan B, Britton RA. The antimicrobial compound reuterin (3-hydroxypropionaldehyde) induces oxidative stress via interaction with thiol groups. Microbiology. 2010 Jun 1;156(6):1589–99.

302. Cleusix V, Lacroix C, Vollenweider S, Duboux M, Le Blay G. Inhibitory activity spectrum of reuterin produced by Lactobacillus reuteri against intestinal bacteria. BMC Microbiol. 2007 Dec;7(1):101.

303. Carey MC, Small DM, Bliss CM. Lipid Digestion and Absorption. Annu Rev Physiol. 1983 Oct;45(1):651–77.

304. Ridlon JM, Kang DJ, Hylemon PB, Bajaj JS. Bile acids and the gut microbiome. Curr Opin Gastroenterol. 2014 May;30(3):332–8.

305. Chiang JYL. Bile Acid Metabolism and Signaling. In: Terjung R, editor. Comprehensive Physiology [Internet]. 1st ed. Wiley; 2013 [cited 2022 Jan 6]. p. 1191–212. Available from: https://onlinelibrary.wiley.com/doi/10.1002/cphy.c120023

306. Payne C. Hydrophobic bile acids, genomic instability, Darwinian selection, and colon carcinogenesis. Clin Exp Gastroenterol. 2008 Dec;19.

307. Bayerdörffer E, Mannes GA, Richter WO, Ochsenkühn T, Wiebecke B, Köpcke W, et al. Increased serum deoxycholic acid levels in men with colorectal adenomas. Gastroenterology. 1993 Jan;104(1):145–51.

308. Bayerdörffer E, Mannes GA, Ochsenkühn T, Dirschedl P, Paumgartner G. Variation of serum bile acids in patients with colorectal adenomas during a one-year follow-up. Digestion. 1994;55(2):121–9.

309. Reddy BS, Wynder EL. Metabolic epidemiology of colon cancer:Fecal bile acids and neutral sterols in colon cancer patients and patients with adenomatous polyps. Cancer. 1977 Jun;39(6):2533–9.

310. Bernstein H, Holubec H, Bernstein C, Ignatenko N, Gerner E, Dvorak K, et al. Unique dietary-related mouse model of colitis: Inflamm Bowel Dis. 2006 Apr;12(4):278–93.

311. Cao H, Xu M, Dong W, Deng B, Wang S, Zhang Y, et al. Secondary bile acid-induced dysbiosis promotes intestinal carcinogenesis: Bile acid, dysbiosis and CRC. Int J Cancer. 2017 Jun 1;140(11):2545–56.

312. Araki Y, Katoh T, Ogawa A, Bamba S, Andoh A, Koyama S, et al. Bile acid modulates transepithelial permeability via the generation of reactive oxygen species in the Caco-2 cell line. Free Radic Biol Med. 2005 Sep;39(6):769–80.

313. Crowley-Weber CL. Development and molecular characterization of HCT-116 cell lines resistant to the tumor promoter and multiple stress-inducer, deoxycholate. Carcinogenesis. 2002 Dec 1;23(12):2063–80.

314. Ding S, Chi MM, Scull BP, Rigby R, Schwerbrock NMJ, Magness S, et al. High-Fat Diet: Bacteria Interactions Promote Intestinal Inflammation Which Precedes and Correlates with Obesity and Insulin Resistance in Mouse. Gaetani S, editor. PLoS ONE. 2010 Aug 16;5(8):e12191.

315. Kim K-A, Gu W, Lee I-A, Joh E-H, Kim D-H. High Fat Diet-Induced Gut Microbiota Exacerbates Inflammation and Obesity in Mice via the TLR4 Signaling Pathway. Chamaillard M, editor. PLoS ONE. 2012 Oct 16;7(10):e47713.

316. Liu Z, Brooks RS, Ciappio ED, Kim SJ, Crott JW, Bennett G, et al. Diet-induced obesity elevates colonic TNF-α in mice and is accompanied by an activation of Wnt signaling: a mechanism for obesity-associated colorectal cancer. J Nutr Biochem. 2012 Oct;23(10):1207–13.

317. Martinez-Medina M, Denizot J, Dreux N, Robin F, Billard E, Bonnet R, et al. Western diet induces dysbiosis with increased *E coli* in CEABAC10 mice , alters host barrier function favouring AIEC colonisation. Gut. 2014 Jan;63(1):116–24.

318. Paik J, Fierce Y, Treuting PM, Brabb T, Maggio-Price L. High-Fat Diet-Induced Obesity Exacerbates Inflammatory Bowel Disease in Genetically Susceptible Mdr1a−/− Male Mice. J Nutr. 2013 Aug 1;143(8):1240–7.

319. Kim I-W, Myung S-J, Do MY, Ryu Y-M, Kim MJ, Do E-J, et al. Western-style diets induce macrophage infiltration and contribute to colitis-associated carcinogenesis: Western-style diets induce colon cancer. J Gastroenterol Hepatol. 2010 Nov;25(11):1785–94.

320. Hildebrandt MA, Hoffmann C, Sherrill–Mix SA, Keilbaugh SA, Hamady M, Chen Y, et al. High-Fat Diet Determines the Composition of the Murine Gut Microbiome Independently of Obesity. Gastroenterology. 2009 Nov;137(5):1716-1724.e2.

321. Xiao L, Sonne SB, Feng Q, Chen N, Xia Z, Li X, et al. High-fat feeding rather than obesity drives taxonomical and functional changes in the gut microbiota in mice. Microbiome. 2017 Dec;5(1):43.

322. Tan R, Dong H, Chen Z, Jin M, Yin J, Li H, et al. Intestinal Microbiota Mediates High-Fructose and High-Fat Diets to Induce Chronic Intestinal Inflammation. Front Cell Infect Microbiol. 2021 Jun 16;11:654074.

323. Cani PD, Neyrinck AM, Fava F, Knauf C, Burcelin RG, Tuohy KM, et al. Selective increases of bifidobacteria in gut microflora improve high-fat-diet-induced diabetes in mice through a mechanism associated with endotoxaemia. Diabetologia. 2007 Nov;50(11):2374–83.

324. Fang W, Xue H, Chen X, Chen K, Ling W. Supplementation with Sodium Butyrate Modulates the Composition of the Gut Microbiota and Ameliorates High-Fat Diet-Induced Obesity in Mice. J Nutr. 2019 May 1;149(5):747–54.

325. Weisburger JH, Reddy BS, Narisawa T, Wynder EL. Germ-Free Status and Colon Tumor Induction by N-Methyl-N'-Nitro-N-Nitrosoguanidine. Exp Biol Med. 1975 Apr 1;148(4):1119–21.

326. Vannucci L, Stepankova R, Kozakova H, Fiserova A, Rossmann P, Tlaskalova-Hogenova H. Colorectal carcinogenesis in germ-free and conventionally reared rats: different intestinal environments affect the systemic immunity. Int J Oncol. 2008 Mar;32(3):609–17.

327. Yi P, Li L. The germfree murine animal: An important animal model for research on the relationship between gut microbiota and the host. Vet Microbiol. 2012 May;157(1–2):1–7.

328. Haghi F, Goli E, Mirzaei B, Zeighami H. The association between fecal enterotoxigenic B. fragilis with colorectal cancer. BMC Cancer. 2019 Dec;19(1):879.

329. Swidsinski A, Khilkin M, Kerjaschki D, Schreiber S, Ortner M, Weber J, et al. Association between intraepithelial Escherichia coli and colorectal cancer. Gastroenterology. 1998 Aug;115(2):281–6.

330. Dejea CM, Fathi P, Craig JM, Boleij A, Taddese R, Geis AL, et al. Patients with familial adenomatous polyposis harbor colonic biofilms containing tumorigenic bacteria. Science. 2018 Feb 2;359(6375):592–7.

331. Rubinstein MR, Baik JE, Lagana SM, Han RP, Raab WJ, Sahoo D, et al. *Fusobacterium nucleatum* promotes colorectal cancer by inducing Wnt/β-catenin modulator Annexin A1. EMBO Rep [Internet]. 2019 Apr [cited 2022 Jan 12];20(4). Available from: https://onlinelibrary.wiley.com/doi/10.15252/embr.201847638

332. Arthur JC, Perez-Chanona E, Mühlbauer M, Tomkovich S, Uronis JM, Fan T-J, et al. Intestinal Inflammation Targets Cancer-Inducing Activity of the Microbiota. Science. 2012 Oct 5;338(6103):120–3.

333. Wu S, Rhee K-J, Albesiano E, Rabizadeh S, Wu X, Yen H-R, et al. A human colonic commensal promotes colon tumorigenesis via activation of T helper type 17 T cell responses. Nat Med. 2009 Sep;15(9):1016–22.

334. Chung L, Thiele Orberg E, Geis AL, Chan JL, Fu K, DeStefano Shields CE, et al. Bacteroides fragilis Toxin Coordinates a Pro-carcinogenic Inflammatory Cascade via Targeting of Colonic Epithelial Cells. Cell Host Microbe. 2018 Feb;23(2):203-214.e5.

335. Gao Z, Guo B, Gao R, Zhu Q, Qin H. Microbiota disbiosis is associated with colorectal cancer. Front Microbiol [Internet]. 2015 Feb 2 [cited 2021 Dec 22];6. Available from: http://journal.frontiersin.org/Article/10.3389/fmicb.2015.00020/abstract

336. Geng J, Fan H, Tang X, Zhai H, Zhang Z. Diversified pattern of the human colorectal cancer microbiome. Gut Pathog. 2013 Dec;5(1):2.

337. Chen W, Liu F, Ling Z, Tong X, Xiang C. Human Intestinal Lumen and Mucosa-Associated Microbiota in Patients with Colorectal Cancer. Moschetta A, editor. PLoS ONE. 2012 Jun 28;7(6):e39743.

338. Wong SH, Zhao L, Zhang X, Nakatsu G, Han J, Xu W, et al. Gavage of Fecal Samples From Patients With Colorectal Cancer Promotes Intestinal Carcinogenesis in Germ-Free and Conventional Mice. Gastroenterology. 2017 Dec;153(6):1621-1633.e6.

339. Schulz MD, Atay Ç, Heringer J, Romrig FK, Schwitalla S, Aydin B, et al. High-fat-diet-mediated dysbiosis promotes intestinal carcinogenesis independently of obesity. Nature. 2014 Oct;514(7523):508–12.

340. Liu T, Guo Z, Song X, Liu L, Dong W, Wang S, et al. High-fat diet-induced dysbiosis mediates MCP-1/CCR2 axis-dependent M2 macrophage polarization and promotes intestinal adenoma-adenocarcinoma sequence. J Cell Mol Med. 2020 Feb;24(4):2648–62.

341. Yang J, Wei H, Zhou Y, Szeto C-H, Li C, Lin Y, et al. High-Fat Diet Promotes Colorectal Tumorigenesis Through Modulating Gut Microbiota and Metabolites. Gastroenterology. 2022 Jan;162(1):135-149.e2.

342. Kastl AJ, Terry NA, Wu GD, Albenberg LG. The Structure and Function of the Human Small Intestinal Microbiota: Current Understanding and Future Directions. Cell Mol Gastroenterol Hepatol. 2020;9(1):33–45.

343. Anders JL, Moustafa MAM, Mohamed WMA, Hayakawa T, Nakao R, Koizumi I. Comparing the gut microbiome along the gastrointestinal tract of three sympatric species of wild rodents. Sci Rep. 2021 Dec;11(1):19929.

CHAPTER 2. OBESOGENIC DIETS INFLUENCE GUT MICROBIOTA COMPOSITION AND INTESTINAL PERMEABILITY

2.1 Abstract

Obesity is a risk factor for a variety of chronic diseases such as diabetes and colorectal cancer. The gut microbiota, which is different in obese individuals compared to lean, has been shown to significantly influence many of these health outcomes. To better understand obesity-associated gut microbiota and potential effects on colonic health we examined the effect of diet and/or obesity on gut microbiota composition and intestinal permeability. We hypothesized that diet and obesity would influence gut microbiota composition and that obese mice would have worse gut barrier function relative to lean mice, regardless of diet. For fifteen weeks, mice were fed a low-fat diet (LFD), high fat diet (HFD) or western diet (WD) *ad libitum* or in controlled amounts to generate lean and obese mice on each diet. Gut microbial analysis demonstrated that the effects of obesogenic diets on microbial communities was dependent on whether the mouse was lean or obese. Richness was higher in lean mice on the WD relative to lean mice on the HFD, but no analogous difference was present between obese mice on the WD and HFD. In addition, microbial communities were more distinct between lean mice on the HFD and LFD, compared to those of obese mice on the HFD and WD. This pattern continued in intestinal permeability as lean mice on the HFD and WD had significantly better barrier function relative to lean-LFD mice, while there were no differences among all three obese groups. Lean mice on obesogenic diets were enriched in microbes that may be beneficial in protecting the colon such as *Coprobacillus,* and obese mice on obesogenic diets were enriched in microbes that may promote intestinal permeability such as those in the *Coriobacteriaceae* and the *Erysipelotrichaceae* families. Collectively, we show that diet and obesity have significant effects on gut microbiota composition and intestinal permeability, and that certain microbes may be important in mediating this outcome.

2.2 Introduction

Models based on current obesity rates in the United States predict that by 2030, 50% of adults will be obese and 25% will be severely obese (1,2). Obesity is a multifaceted condition that is associated with increased risk for many leading causes of death in the US such as cardiovascular

disease (3). There are various changes that occur in obese patients that can contribute to poor health, including the gut microbiota. Shifts in gut microbial communities associated with obesity have been linked to conditions such as increased inflammation, metabolic endotoxemia, and insulin resistance (4–8).

The colon harbors most of the trillions of bacteria present in and on the human body (9). While a variety of factors such as host genetics play a role in shaping the gut microbiota, diet is generally a strong force (10–17). As most undigested nutrients in the colon are available for bacterial action, dietary effects on substrate availability influence the number and types of microorganisms that survive and proliferate. Obesity is largely driven by excess consumption of diets high in saturated fat, refined sugars, and animal protein (18–21). While this suggests that diet is the primary factor responsible for obesity associated shifts in gut microbiota composition, other changes present in the obese state may also have an influence. Two research groups took advantage of mouse models that had differences in susceptibility to obesity due to genetic modification. In both studies, diet was the major predictor, explaining 28% of the variation in microbial communities (22,23). However, genotype was the second major predictor, explaining 7% or 19% of the variation in gut microbiota composition. Any effects that could have been attributed to obesity were likely confounded by the genotype-obesity relationship inherent in the animal models. Considering this limitation, it is still unclear if and/or to what extent obesity and diet influence gut microbiota composition.

Other than its causal link to the development of obesity, the gut microbiota has been shown to be an important factor in colonic health (24,25). In homeostasis, commensal microbes and their metabolites activate anti-inflammatory pathways in the colon that promote tissue repair to prevent intestinal permeability (26–30). Obesity-associated changes to the gut microbiota have been shown to disturb these processes and are linked to increased inflammation and decreased gut barrier function (4,31,32). However, obesity-related conditions like chronic inflammation also influence intestinal health (33). As a result, effects of the gut microbiota on colonic health outcomes such as leaky gut are confounded by the development of obesity.

In this study, we aimed to assess how diet and/or obesity influence gut microbiota composition and intestinal permeability. Since diet is a key factor in promoting obesity and shaping gut microbial communities, two different obesogenic diets, a high fat diet (60% fat) and a western diet (40% fat, 30% sucrose) were used. To gain insight into potential microbial-colonic health

connections, markers of intestinal barrier function were measured. We hypothesized that obesogenic diets and obesity would significantly influence gut microbiota composition and be associated with reduced intestinal permeability.

2.3 Methods

2.3.1 Study design and animal husbandry

Animal studies and procedures were approved and monitored by Institutional Animal Care and Use Committee at Purdue University. Mice were individually housed in a humidity and temperature-controlled facility with a 12 hr light-dark cycle. Six-week-old male wildtype C57BL/6J mice (n=20/group) and *ob/ob* mice, C57BL/6J background (n=20) were purchased from Jackson Laboratories and fed a low-fat diet (LFD, 10 kcal% fat, #D12450J, Research Diets, Inc., New Brunswick, NJ, USA) *ad libitum* for one week of acclimatization. *Ob/ob* mice express a mutation in the leptin gene that induces hyperphagia. This group was maintained on the LFD throughout the study and was included to generate an obese-LFD group. C57BL/6J mice were then randomized into 5 diet groups (see table 2.1 for diet composition): (i) LFD, (ii) high-fat diet (HFD, 60% of kcals from fat), (iii) western diet (WD, 40% kcals from fat, 30% kcals from sucrose), (iv) pair-fed HFD (lean-HFD), and (v) pair-fed WD (lean-WD). Pair-fed mice received a daily ration of HFD or WD that was isocaloric to the number of kilocalories (kcals) consumed by the lean-LFD control group a week prior. However, previous research indicated that pair-fed mice on obesogenic diets still gain more weight relative to a LFD control (34). We monitored and recorded body weight twice a week and adjusted the number of kcals consumed so that lean-HFD and lean-WD had comparable weight to lean-LFD. Mice were pair-fed starting at week 6. After 15 weeks on these dietary interventions, mice were euthanized with CO_2 and cervical dislocation. Body fat percentage was measured using EchoMRI^{TM} on the same day as euthanization. Fresh fecal pellets were collected at week 15 post-dietary intervention, then stored in the -80°C freezer.

2.3.2 Gut microbiota analysis using 16s rRNA gene sequencing

Genomic DNA was extracted from fecal contents using a bead-beating protocol (35). Briefly, ~ 25 mg of fecal pellet sample was suspended in a solution containing 500 µl of extraction

buffer [200 mM Tris:HCl (pH 8.0), 200 mM NaCl, 20 mM EDTA], 210 µl of 20% SDS, 500 µl of Invitrogen™ UltraPure™ phenol:chloroform:isoamyl alcohol (cat# 15593049, Waltham, MA), and 500 µl of 0.1-mm diameter zirconia/silica beads. Samples were mechanically disrupted for 3 min at 3400 RPM under room temperature using a bead beater (Mini-beadbeater 96, BioSpec, Barlesville, OK), followed by centrifugation. The aqueous phase was recovered using 60 µl of 3 M NaAcetate and 600 uL isopropanol precipitation. QIAquick 96-well PCR Purification Kit (Qiagen, cat# 28181, Germantown, MD) was used to remove contaminants. Isolated DNA was eluted in 10 mM TE (pH 8.0) buffer and was stored at −20 °C until further use.

Amplification of the 16S rRNA gene targeting the V4 region was generated from extracted genomic DNA by PCR using unique 8-bp barcodes on the forward and reverse primers and fused with Illumina sequencing adapters (36). Each sample was amplified in duplicate in a reaction volume of 25 uL using KAPA HiFi HotStart DNA polymerase (KAPA Biosystems, KK2602, Wilmington, MA), 10 µM of each primer, and 12.5 ng of genomic DNA. PCR was carried out under the following conditions: initial denaturation for 3 min at 95°C, followed by 25 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 55°C and elongation for 30 s at 72°C, and a final elongation step for 5 min at 72°C. PCR products were purified with Sera-Mag Select beads, left side size selection (Cytiva, Shrewsbury, MA, 29343052). Equimolar amounts of each sample were pooled and then sequenced using the Illumina MiSeq platform with V2 chemistry (2 × 250 bp) at the Bindley Core for Genomics at Purdue University.

Sequences were processed using a QIIME 2 pipeline (37). Demultiplexed 250 bases paired-end sequences were imported using Casava 1.8 format and denoised using DADA2 (38,39) to obtain an amplicon sequence variant (ASV) table. ASVs present in less than 20 samples were discarded. This condition resulted in the lowest total frequency for a feature to be 164. A naïve Bayes taxonomy classifier was trained using Greengenes (40) reference sequences (clustered at 99% similarity) to taxonomically annotate ASVs. An even sampling depth of 11,730 sequences per sample was used for assessing alpha- and beta-diversity measures. Kruskal-Wallis followed by Conover Iman was used to calculate differences among groups for alpha diversity metrics (Shannon diversity index, Faith's phylogenetic diversity (PD), observed taxonomic units (OTUs), and Pielou's Evenness). Beta-diversity was measured using unweighted and weighted UniFrac distances and visualized using principal coordinates analysis (PCoA) (41). Differences in UniFrac distances between microbiota communities were tested by pairwise PERMANOVA using QIIME2

beta-group-significance command with the -p-pairwise parameter. P-values corrected for multiple comparisons using Benjamini-Hochberg is denoted by q-values (42). Linear discriminant analysis (LDA) effect size (LEfSe Galaxy Version 1.0) was performed to identify differentially abundant taxa using default p value ($\alpha = 0.05$), LDA score of 2.0, and one against all for multi-class analysis strategy (43).

2.3.3 Gut barrier function assessment using FITC-dextran assay

Fourteen-weeks after dietary treatments, intestinal barrier function was assessed in a subgroup of mice (n=10 mice/treatment group). Mice were fasted for 6 hours then dosed with 500 mg/kg body weight of 4,000 Da fluorescent FITC-dextran (Sigma, cat# 46944, Burlington, MA) via oral gavage. After four hours, serum was collected by centrifuging blood acquired through nonterminal submandibular bleed at 4°C, 2000 x g for 10 minutes. Serum was diluted in 1:1 ratio with PBS and analyzed for FITC-dextran concentration with a fluorescence spectrophotometer (Biotek Instruments Synergy LX plate reader, cat# BTSLXFA) at excitation wavelength of 485 nm and emission wavelength of 535 nm. A standard curve using FITC-dextran serially diluted in PBS (8000 ng/ml - 0 ng/ml) was used to determine FITC-dextran concentration in serum.

2.3.4 Histological assessment of the intestinal barrier

At euthanization, colons were resected, cut open and laid flat, cleaned with PBS, rolled from distal to proximal end to form a swiss-roll, and placed in a cassette. They were submerged in 10% neutral buffered formalin for 48 hours and stored in 70% ethanol. Samples were submitted to the Histology Research Laboratory at Purdue University for assessment of mucin-2 (MUC2), a mucus protein secreted by goblet cells that protects the epithelium, and zonula occludin-1 (ZO1), a tight junction protein. Samples were processed overnight on the Sakura Tissue-Tek VIP 6 vacuum infiltration tissue processor then embedded in paraffin wax molds and sectioned onto slides at 4 um thickness. After sectioning, slides were incubated for one hour at 57°C, deparaffinized, and then put through the antigen retrieval process in a decloaker at 95°C for 20 minutes. For tissues intended to be stained with MUC2 (Novus Bio, NBP1-31231, Littleton, CO), the antigen retrieval buffer used was EDTA, and Diva decloaker for ZO-1 (Abcam, cat# ab221546, Waltham, MA). Slides were then rinsed in Tris EDTA buffer, marked with hydrophobic pen and

transferred to the Biocare intelliPATH automatic stainer. Tissues were first blocked with 3% hydrogen peroxide, then with 2.5% normal goat serum. After a buffer rinse, primary antibody was dispensed, both at a 1:500 dilution. MUC2 incubated for 60 minutes, and ZO-1 incubated for 30 minutes. Slides were double rinsed with buffer and stained with the ImmPRESS HRP goat anti-rabbit secondary antibody for 30 minutes. Following an additional double rinse, the 3,3'-diaminobenzdine (DAB) chromogen was applied for 5 minutes. Lastly, slides were counterstained with hematoxylin, dehydrated, and cover-slipped with resinous mounting media. Images were uploaded to Aperio and analyzed with ImageJ software (44). Tissue sections were outlined by hand and percentage of negative, weak, medium, and strong staining intensity was determined using a macro designed in collaboration with a histopathologist.

2.3.5 Statistical Analysis

All statistical analysis was performed using R version 4.1.2 (2021-11-01) (45) or QIIME2 (2021.4) (37). Body weight, body fat percent, and kilocalories consumed were assessed using a 2-way ANOVA followed by Tukey HSD. Differences in FITC-dextran concentration was assessed with gamma GLM, and MUC2 and ZO-1 were analyzed with beta regression. Outcomes with q-values or p-values < 0.05 are considered statistically significant.

Table 2.1 Diet composition for low fat diet (LFD), high fat diet (HFD), and western diet (WD)

	LFD		HFD		WD	
	gm%	kcal%	gm%	kcal%	gm%	kcal%
Protein	19	20	26	20	23	20
Carbohydrate	67	70	26	20	46	40
Fat	4	10	35	60	20	40
Total		100		100		100
kcal/gm	3.85		5.24		4.58	

Ingredient	gm	kcal	gm	kcal	gm	kcal
Casein	200	800	200	800	200	800
L-Cystine	3	12	3	12	3	12
Corn starch	506.2	2024.8	0	0	0	0
Maltodextrin 10	125	500	125	500	50	200
Sucrose	72.8	291.2	68.8	275	344	1376
Cellulose, BW200	50	0	50	0	50	0
Soybean Oil	25	225	25	225	25	225
Lard	20	180	245	2205	156	1404
Mineral Mix, S10026	10	0	10	0	10	0
DiCalcium Phosphate	13	0	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0	5.5	0
Potassium Citrate, 1 H2O	16.6	0	16.5	0	16.5	0
Vitamin Mix, V10001	10	40	10	40	10	40
Choline Bitartrate	2	0	2	0	2	0
FD&C Yellow Dye #5	0.04	0	0	0	0.025	0
FD&C Red Dye #40	0.01	0				
FD&C Blue Dye #1			0.05	0	0.025	0
total	1055.05	4032	773.85	4057	885.05	4057

2.4 Results

2.4.1 Obesogenic diets promote weight gain and fat gain

Mice were weighed twice weekly and body fat was measured once using EchoMRI™ on the day of euthanasia. Mice on the HFD and WD *ad libitum* (obese-HFD, obese-WD) gained a significant amount of weight (Fig 2.1A) and body fat (Fig 2.1B) relative to their lean counterparts lean-HFD and lean-WD. Lean-HFD and lean-WD mice maintained their weight comparable to lean-LFD. Twice-a-week body weight measurements allowed as needed adjustment of kcal to maintain a lean phenotype on an obesogenic diet (Fig 2.1C). Obese-LFD (*ob/ob)* mice were significantly heavier throughout the study and had significantly higher body fat at week 15 relative to all other groups. At week 1, obese-LFD consumed significantly more kcals compared to the 5 other groups, but by week 15, this measurement was comparable to the obese-HFD and obese-WD.

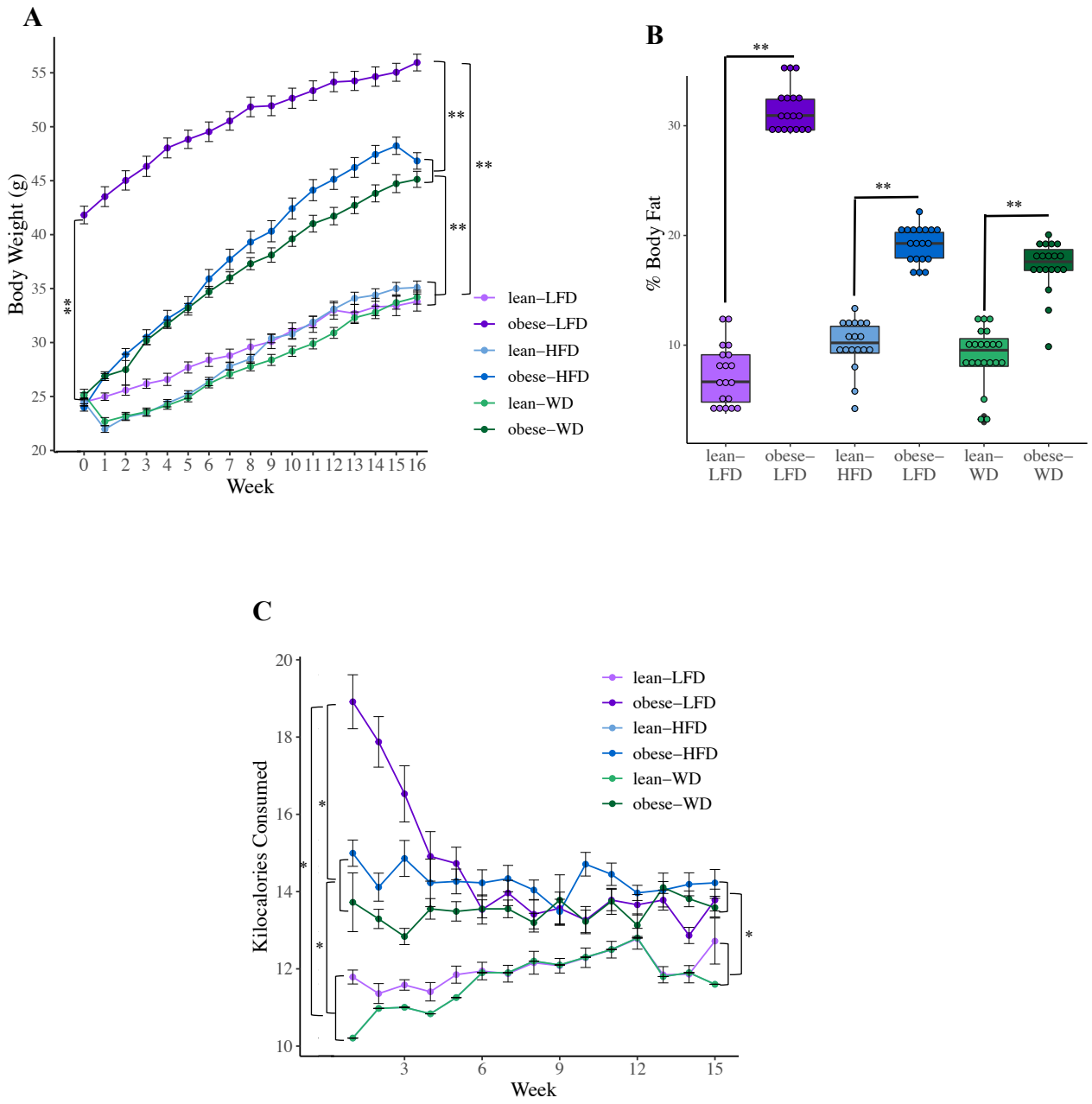

Figure 2.1 Body weight, body fat and kilocalories consumed

Male C57BL/6J mice consumed *ad libitum* a LFD (lean-LFD), HFD (obese-HFD), or WD (obese-WD), or given controlled portions of HFD (lean-HFD) and WD (lean-WD) to prevent weight gain. Male *ob/ob* consuming a LFD *ad libitum* (obese-LFD) were included as the obese counterpart to lean-LFD. (A) Body weights were tracked for 15 weeks (B) prior to euthanasia, body fat percent was measured using EchoMRI™ (C) weekly monitoring and recording of kcal consumed. n=18-20/group. Significance was calculated using a 2-way ANOVA followed by Tukey HSD * $p< 0.05$, **$p<0.01$

2.4.2 Alpha diversity was significantly influenced by obesity and diet

Using four alpha diversity metrics, Faith's PD, observed features, Pielou's evenness, and Shannon entropy, the richness and abundance of microbiota in a fecal sample collected at week fifteen was assessed. Faith's PD incorporates phylogenetic relationship; observed features is the number of unique sequences identified in a sample; Pielou's evenness assesses abundance; Shannon entropy considers richness and abundance. Diet and obesity were significant predictors of richness in Faith's PD (Fig 2.2A) and observed features (Fig 2.2B). Obese groups had significantly less richness as measured by Faith's PD and observed features compared to their lean counterparts in all diets. There was a significant main effect of diet in which LFD groups had significantly lower richness scores compared to HFD and WD. Lean-HFD had significantly less richness compared to lean-WD, but there was no difference between obese-HFD and obese-WD. (Fig 2.2A-B). Because we used nonparametric methods to compare groups, we could not include an interaction term in our model. However, using the Conover-Iman test, we could compare the estimates for the difference between lean and obese mice for each diet group. For Faith's PD, the difference between lean and obese LFD was 3.8, between lean and obese HFD was 2.5, and between lean and obese WD was 4. These results suggested that there was an interaction between obesity and HFD. To gain insight into whether this interaction was true, we used linear regression. Although this data does not satisfy all assumptions for linear regression, by comparing adjusted r^2 values, we could determine whether including the interaction term explained a higher percentage of the variation in the data. The model including the interaction term calculated a significant interaction between obesity and HFD ($p=0.03$) and had a higher adjusted r^2 relative to the model without the interaction term, 0.41 and 0.399 respectively. Together, these results indicate that there is an interaction between obesity and diet whereby developing obesity on the HFD had a smaller effect on Faith's PD score relative to developing obesity on the LFD or WD. The same result was not detected in observed features.

We did not observe a significant effect of obesity on Shannon's Entropy (Fig 2.2C) and Pielou's Evenness (Fig 2.2D), but for both metrics, diet had a significant effect on the outcome. In both Shannon's entropy and Pielou's Evenness, mice on the LFD had lower scores compared to mice on the WD and HFD, and mice on the WD had scores that were significantly higher compared to the HFD mice.

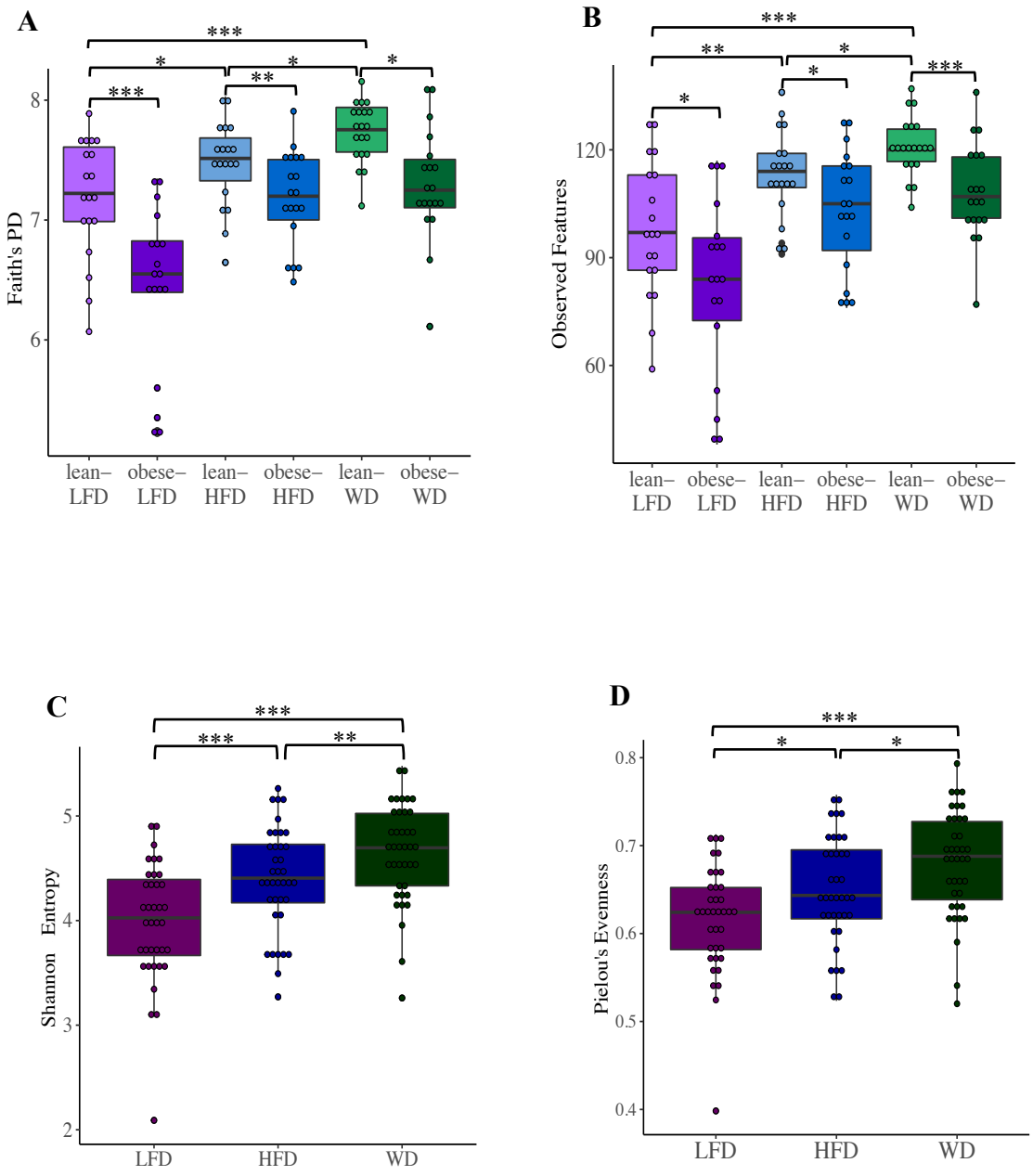

Figure 2.2 Alpha diversity in fecal samples after 15 weeks on dietary treatments

Using 16S rRNA gene sequencing, alpha diversity was measured in fecal samples collected from C57BL/6J male mice fed restricted amounts or fed *ad libitum* a low-fat diet (lean-LFD, obese-LFD), high fat diet (lean-HFD, obese-HFD) or western diet (lean-WD, obese-WD) for 15 weeks. (A) Faith's PD (B) observed features (C) Shannon Entropy (D) Pielou's Evenness. n=18-20/group. LFD=purple, HFD=blue, WD=green, light shades = lean, dark shades = obese. Data was analyzed using Kruskal-Wallis followed by Conover-Iman * $p<0.05$, ** $p<0.01$, ***$p<0.001$

2.4.3 Beta-diversity in fecal microbiota was significantly influenced by obesity and diet

Differences in fecal microbiota composition based on obesity status and/or diet were calculated using weighted and unweighted UniFrac distances. Principal coordinates analysis (PCoA) was used to visualize differences between communities in which a point represents a summary of the composition of the gut microbiota for one sample, incorporating relative abundance and phylogenetic relationship among microbes. The greater the distance between two points indicates the greater the dissimilarity between the gut microbiotas. After fifteen weeks, diet and obesity had significant main effects on microbial composition ($q<0.05$) based on both weighted and unweighted UniFrac distances. Only weighted UniFrac PcoAs are plotted. Given sample overlap between lean and obese (Fig 2.3A) and among diets (Fig 2.3B), there were similarities in microbial composition, particularly between the HFD and WD groups. To visualize distances based on diet and obesity, groups were colored such that LFD is in purple, HFD is in blue, and WD is in green with darker shades representing the obese group and lighter shades representing the lean counterpart (Fig 2.3C). There were statistical differences among all six groups ($q< 0.05$), even though differential clustering is not apparent among all treatment groups. We observed that the lean-HFD and lean-WD appeared more clustered and defined from each other relative to the obese-HFD and obese-WD, which appeared more variable and visually not as distinct from each other. Interestingly, based on the PcoA of unweighted UniFrac distances, the obese-HFD and obese-WD groups were visually more differentially clustered (data not shown), suggesting that differences in taxa abundance increases variability in the HFD and WD groups.

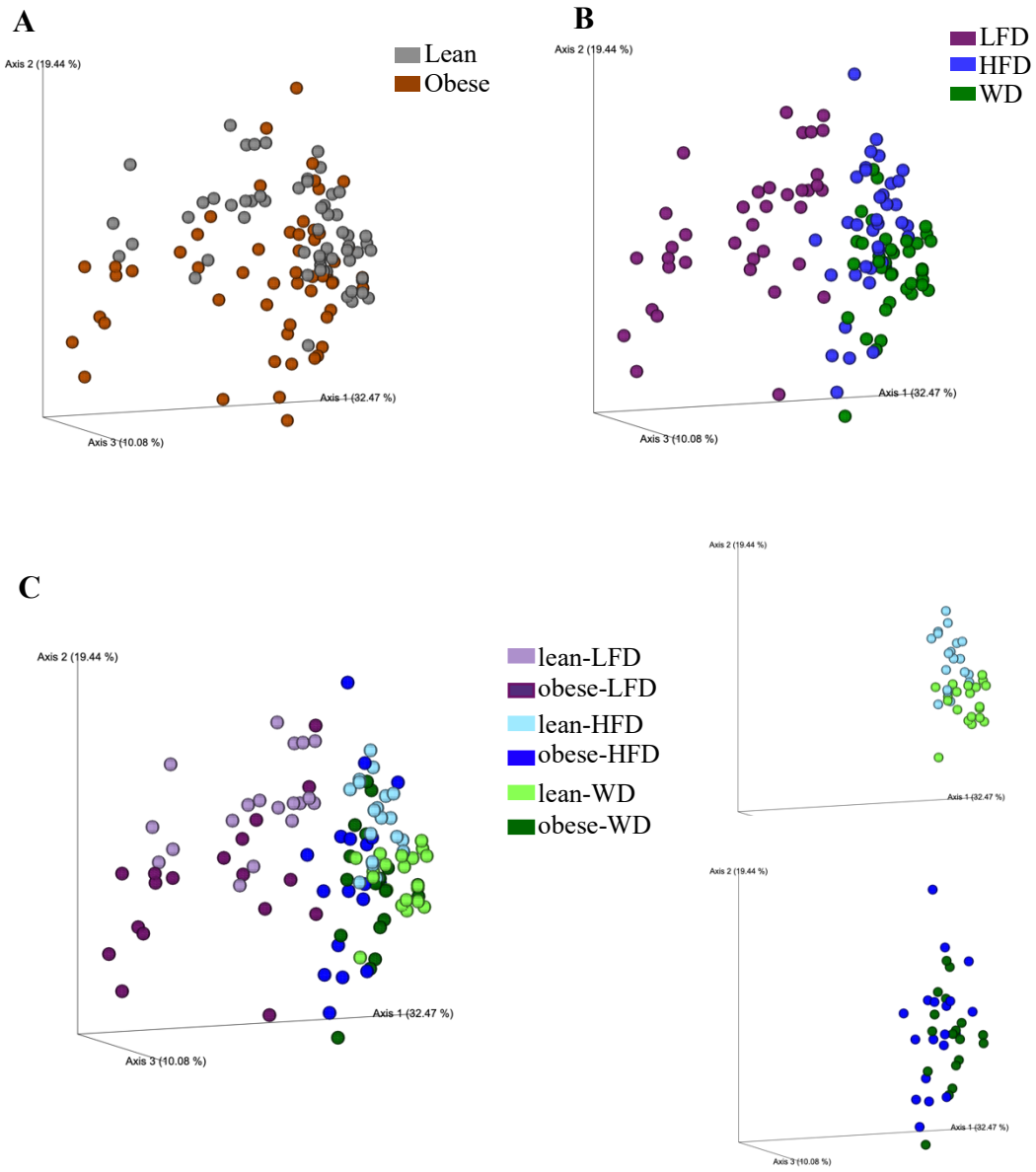

Figure 2.3 Beta diversity in fecal samples after 15 weeks on dietary treatments

Using 16S rRNA gene sequencing, beta diversity was measured in fecal samples collected from C57BL/6J male mice fed restricted amounts or fed *ad libitum* a low-fat diet (lean-LFD, obese-LFD), high fat diet (lean-HFD, obese-HFD) or western diet (lean-WD, obese-WD) for 15 weeks. Principal coordinate analysis plotted based on weighted UniFrac distances and colored by (A) obesity, brown=obese, grey=lean (B) diet, purple = LFD, blue = HFD, and green = WD and (C) obesity + diet, light purple = lean-LFD, dark purple=obese-LFD, light blue = lean-HFD, dark blue = obese-HFD, light green = lean-WD, dark green = obese-WD. n= 18-20/group, q=0.001 between lean and obese, LFD and HFD, LFD and WD, HFD and WD, and between all pairings among the 6 groups. Data was analyzed using PERMANOVA followed by pairwise testing, α=0.05.

2.4.4 Obesity associated effects on taxa enrichment is influenced by diet

To assess the effect of obesity status on taxa enrichment, we performed three separate LEfSe analyses comparing lean and obese mice on the LFD (Fig 2.4B), HFD (Fig 2.4C) and WD (Fig 2.4D), and relative abundance was visualized by phyla (Fig 2.4A). Compared to their lean counterpart, Actinobacteria, specifically *Coriobacteriaceae*, was enriched in the obese-HFD and obese-WD groups and Firmicutes was enriched in the obese-LFD group. Enriched Proteobacteria measured in all lean groups compared to their obese counterpart was attributed to *Sutterella*. Compared to their obese counterpart, lean-LFD mice were enriched in Verrucomicrobia, Proteobacteria and Bacteroidetes, lean-HFD mice were enriched in Proteobacteria, and lean-WD were enriched in Proteobacteria, Tenericutes and Verrucomicrobia. Other significantly enriched taxa detected between lean and obese mice on each diet group can be found in Figures 2.4B-D. If obesity status had the same effect across all diets, we would expect considerable overlap in significantly enriched taxa among all diets in the lean group and among all diets in the obese group. This was not the case as seen in table 2.2 and table 2.3 and suggests that there was an interaction between diet and obesity. Since the HFD and WD shared a substantial number of similar taxa in the lean (table 2.2) and obese (table 2.3) groups it seems likely the presence or absence of an obesogenic diet influences microbial enrichment.

To further gain further information about specific diet effects, we identified taxa enriched in exactly one of the three diets in lean mice (table 2.4) and obese mice (table 2.5) based on the LEfSe analysis in Fig 2.4B-D. Lean-LFD had the highest number of single enriched taxa such as *Ruminococcaceae*, *Lactococcus, Turicibacter, Blautia,* and *Clostridium*. In lean-HFD mice, *Adlercreutzia, Dehalobacterium*, and *Butyricicoccus* were the three single taxa and *Lactobacillus* appeared to be only enriched in lean-WD mice (table 2.4). *Allobaculum* was only enriched in obese mice on the LFD, while there were two in obese-HFD, *Blautia* and *Erysipelotrichaceae*, and three in obese-WD, *S24-7*, *Turicibacter*, and *Clostridaceae* (table 2.5).

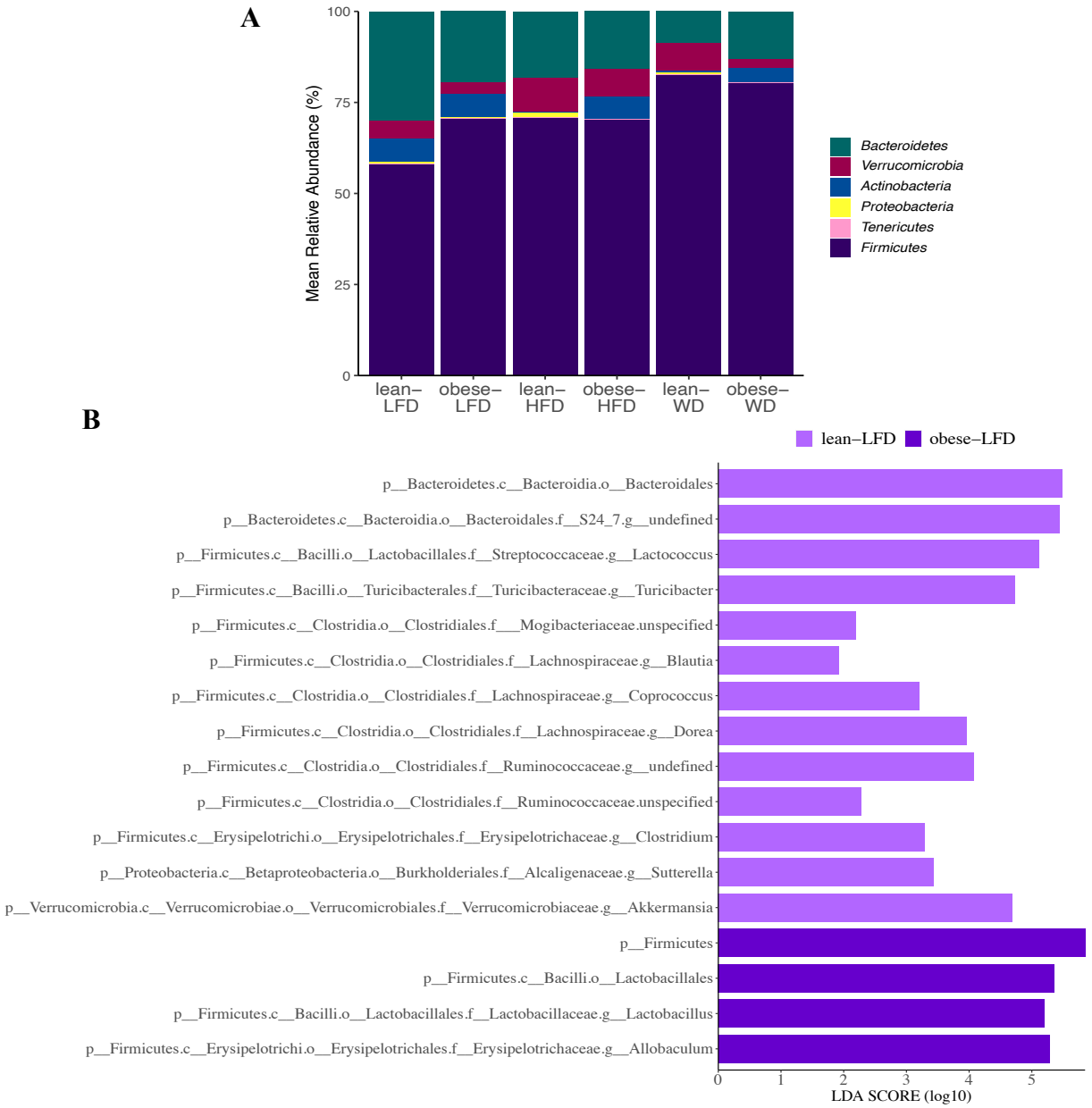

Figure 2.4 Differentially abundant taxa between lean and obese mice on the low-fat diet, high fat diet and western diet

Significantly enriched gut microbes in fecal samples collected from C57BL/6J male mice fed restricted amounts or fed *ad libitum* a low-fat diet (lean-LFD, obese-LFD), high fat diet (lean-HFD, obese-HFD) or western diet (lean-WD, obese-WD) for 15 weeks. (A) Percent mean relative abundance by phyla in each group (B) Linear discriminant analysis of effect size (LEfSe) analysis of significantly enriched taxa measured between lean and obese LFD groups (C) lean and obese HFD groups (D) and lean and obese WD groups. g_undefined indicates there was not enough information in the database to distinguish the specific genus. Unspecified indicates there was not enough information to distinguish past that level of taxonomic classification. Bars indicate effect size of each taxon. n=18-20/group, $p<0.05$

Figure 2.4 continued

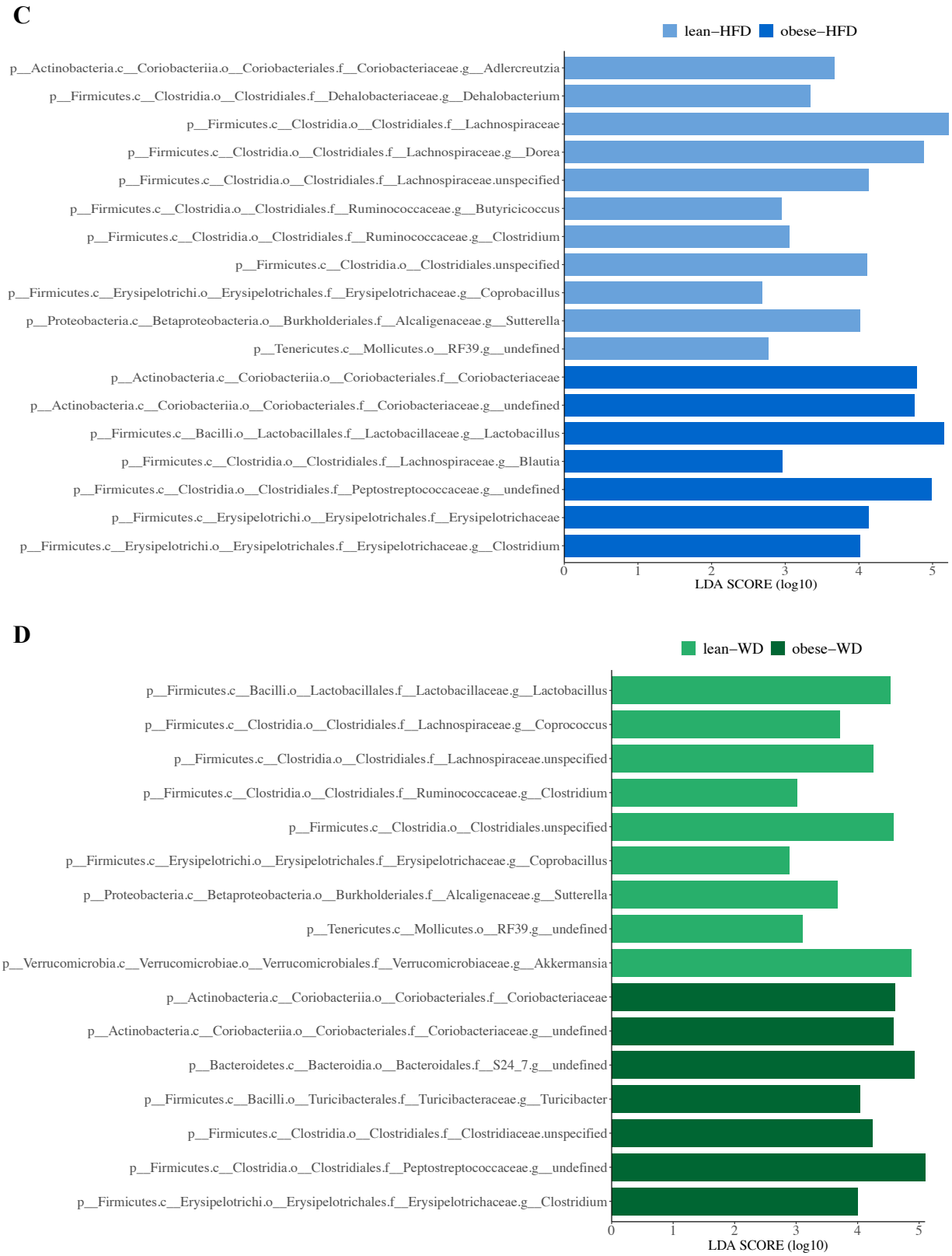

Table 2.2 Significantly enriched taxa shared among lean mice on a low-fat diet (LFD), high fat diet (HFD) and western diet (WD)

16S rRNA sequencing data was used to determine the identity of gut microbiota from fresh fecal samples collected from C57BL/6J male mice that were fed *ad libitum* LFD (lean-LFD), or controlled amounts of HFD (lean-HFD), or WD (lean-WD) to match lean-LFD weight gain for fifteen weeks. Taxa listed were those significantly enriched in lean groups that were also present in at least two of the diet groups based on linear discriminant analysis of effect size (LEfSe) analysis ($p<0.05$)

Lean Mice			
Taxa	**LFD**	**HFD**	**WD**
p__Firmicutes.c__Clostridia.o__Clostridiales. f__Lachnospiraceae.g__Coprococcus	X		X
p__Firmicutes.c__Clostridia.o__Clostridiales. f__Lachnospiraceae.g__Dorea	X	X	
p__Firmicutes.c__Clostridia.o__Clostridiales. f__Lachnospiraceae		X	X
p__Firmicutes.c__Clostridia.o__Clostridiales. f__Ruminococcaceae.g__Clostridium		X	X
p__Firmicutes.c__Clostridia.o__Clostridiales		X	X
p__Firmicutes.c__Erysipelotrichi.o__Erysipelotrichales. f__Erysipelotrichaceae.g__Coprobacillus		X	X
p__Proteobacteria.c__Betaproteobacteria.o__Burkholderiales. f__Alcaligenaceae.g__Sutterella	X	X	X
p__Tenericutes.c__Mollicutes.o__RF39		X	X
p__Verrucomicrobia.c__Verrucomicrobiae.o__Verrucomicrobiales. f__Verrucomicrobiaceae.g__Akkermansia	X		X

Table 2.3 Significantly enriched taxa shared among obese mice on the low-fat diet (LFD), high fat diet (HFD) and western diet (WD)

16S rRNA sequencing data was used to determine the identity of gut microbiota from fresh fecal samples collected from C57BL/6J male mice that were fed *ad libitum* LFD (obese-LFD), or HFD (obese-HFD), or WD (obese-WD) for fifteen weeks. Taxa listed were those significantly enriched in obese groups that were also present in at least two of the diet groups based on linear discriminant analysis of effect size (LEfSe) analysis on 16S rRNA sequencing data, n =18-20/group, $p<0.05$

Obese Mice			
Taxa	**LFD**	**HFD**	**WD**
p__Actinobacteria.c__Coriobacteriia.o__Coriobacteriales. f__Coriobacteriaceae		X	X
p__Actinobacteria.c__Coriobacteriia.o__Coriobacteriales. f__Coriobacteriaceae		X	X
p__Firmicutes.c__Bacilli.o__Lactobacillales .f__Lactobacillaceae.g__Lactobacillus	X	X	
p__Firmicutes.c__Clostridia.o__Clostridiales. f__Peptostreptococcaceae		X	X
p__Firmicutes.c__Erysipelotrichi.o__Erysipelotrichales. f__Erysipelotrichaceae.g__Clostridium		X	X

Table 2.4 Significantly enriched single taxa in lean mice on a low-fat diet (LFD), high fat diet (HFD), and western diet (WD).

16S rRNA sequencing data was used to determine the identity of gut microbiota from fresh fecal samples collected from C57BL/6J male mice that were fed *ad libitum* LFD (lean-LFD), or controlled amounts of HFD (lean-HFD), or WD (lean-WD) to match lean-LFD weight gain for fifteen weeks. Taxa listed were those significantly enriched in the lean group and present in only one diet based on linear discriminant analysis of effect size (LEfSe) analysis, n=18-20 mice/group, $p<0.05$

Lean Mice	
Taxa	**Diet**
p__Actinobacteria.c__Coriobacteriia.o__Coriobacteriales.f__Coriobacteriaceae. g__Adlercreutzia	HFD
p__Firmicutes.c__Clostridia.o__Clostridiales.f__Dehalobacteriaceae. g__Dehalobacterium	HFD
p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae	HFD
p__Firmicutes.c__Clostridia.o__Clostridiales.f__Ruminococcaceae. g__Butyricicoccus	HFD
p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales	LFD
p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__S24_7	LFD
p__Firmicutes.c__Bacilli.o__Lactobacillales.f__Streptococcaceae.g__Lactococcus	LFD
p__Firmicutes.c__Bacilli.o__Turicibacterales.f__Turicibacteraceae.g__Turicibacter	LFD
p__Firmicutes.c__Clostridia.o__Clostridiales.f__Mogibacteriaceae.unspecified	LFD
p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__Blautia	LFD
p__Firmicutes.c__Clostridia.o__Clostridiales.f__Ruminococcaceae	LFD
p__Firmicutes.c__Clostridia.o__Clostridiales.f__Ruminococcaceae	LFD
p__Firmicutes.c__Erysipelotrichi.o__Erysipelotrichales.f__Erysipelotrichaceae. g__Clostridium	LFD
p__Firmicutes.c__Bacilli.o__Lactobacillales.f__Lactobacillaceae.g__Lactobacillus	WD

Table 2.5 Significantly enriched single taxa in obese mice on a low-fat diet (LFD), high fat diet (HFD), and western diet (WD)

16S rRNA sequencing data was used to determine the identity of gut microbiota from fresh fecal samples collected from C57BL/6J male mice that were fed *ad libitum* LFD (obese-LFD), or HFD (obese-HFD), or WD (obese-WD) for fifteen weeks. Taxa listed were those significantly enriched in only one diet group based on linear discriminant analysis of effect size (LEfSe) analysis ($p<0.05$), n=18-20 mice/group.

Obese mice	
Taxa	**Diet**
p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__Blautia	HFD
p__Firmicutes.c__Erysipelotrichi.o__Erysipelotrichales.f__Erysipelotrichaceae	HFD
p__Firmicutes.c__Erysipelotrichi.o__Erysipelotrichales.f__Erysipelotrichaceae. g__Allobaculum	LFD
p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__S24_7	WD
p__Firmicutes.c__Bacilli.o__Turicibacterales.f__Turicibacteraceae. g__Turicibacter	WD
p__Firmicutes.c__Clostridia.o__Clostridiales.f__Clostridiaceae	WD

2.4.5 Lean-HFD and lean-WD display better gut barrier function relative to lean-LFD

The effects of diet and obesity on intestinal permeability via the *in vivo* FITC-dextran test were determined (Fig 2.5A). Results indicated that diet was a significant predictor. HFD and WD groups were associated with lower serum FITC-dextran concentrations indicating less intestinal permeability. Initially, obesity was not a significant predictor. However, since the obese-LFD were genetically modified and were significantly heavier and had higher percent body fat compared to obese-WD and obese-HFD (Fig 2.1A-B), we speculated that genetic-obesity and associated phenotypes may have imparted a different effect relative to mice on the obesogenic diets. After recoding obesity as genetic or diet, results indicated that obesity-diet was associated with a significant increase in of serum FITC-dextran concentration ($p=0.007$). There were no differences in intestinal permeability among obese groups, but lean-HFD and lean-WD mice had less leakiness compared to lean-LFD mice. Notably, there are two potential outliers in the obese-HFD and obese-WD groups. To determine their leverage on the results, we removed the two data points and ran

the same tests. Obesity-diet was no longer a significant predictor. However, based on the histogram, it appears that the distribution of data in the obese-HFD and obese-WD groups is wider, and more variable compared to their lean counterparts. This makes it difficult to determine if these points are true outliers. Since the values of the two potential outliers are not unreasonable, we used the model that included them in the analysis. To assess local components of the colonic barrier that can influence permeability, we next tested whether the proportion of strong staining intensity of MUC2 and ZO-1 were significantly influenced by diet and obesity. Histological signs of changes in barrier function were not detected (Fig 2.5B-C).

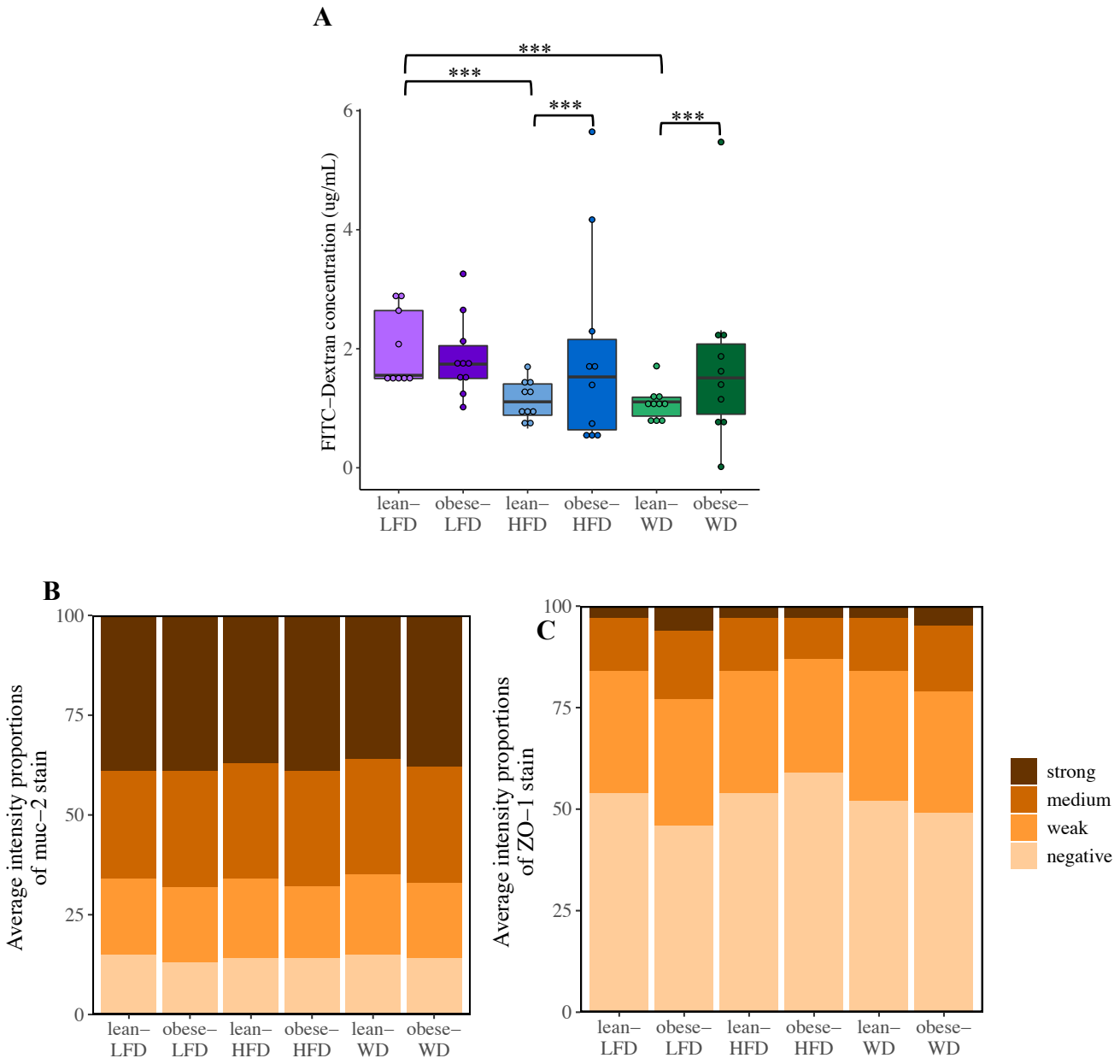

Figure 2.5 Measurements of intestinal barrier integrity

C57BL/6J male mice fed restricted amounts or fed *ad libitum* a low-fat diet (lean-LFD, obese-LFD), high fat diet (lean-HFD, obese-HFD) or western diet (lean-WD, obese-WD). (A) At week 14, intestinal permeability was tested by measuring serum FITC-dextran in 8-10 mice/group. Box and whisker plots have lines for 25th percentile, 75th percentile, and median. Lines extending out represent maximum and minimum values. After 15 weeks, mice were euthanized, colons were swiss-rolled and then fixed in formalin and paraffin embedded. Samples were prepared for staining with (B) MUC2 and (C) ZO-1 (C), n=5/group. Graph represents average proportion of negative, weak, medium, and high staining intensity. Data was analyzed using gamma GLM for FITC-dextran and beta regression for MUC2 and ZO1 ** $p < 0.01$.

2.5 Discussion

In this study, we examined the effect of obesity and two obesogenic diets on gut microbiota composition and intestinal permeability. To account for potential changes to the gut microbiota due to the physiological and metabolic changes associated with obesity, we included a lean counterpart for each obese mouse. This design allowed us to separate the effects of diet and obesity on gut microbiota composition, and to capture results relevant to an obese and lean population. In addition, to gain insight into local effects of the gut microbiota, we assessed gut barrier function through an *in vivo* test and through histological staining of MUC2 and ZO-1 in the colon. Overall, we found that both diet and obesity status influence the gut microbiota and intestinal permeability.

Like other studies, we found that the HFD and WD significantly changed gut microbiota relative to the LFD control (22,23,46–48). For all alpha diversity metrics, there was a significant diet effect. The diets chosen for this study represent unhealthy eating habits that are linked to detrimental conditions that are commonly associated with lower alpha diversity. Contrary to the unhealthy nature of the diets, however, mice on the HFD and WD had significantly higher alpha diversity relative to the LFD, and the WD had significantly higher diversity compared to the HFD. The link between alpha diversity and health outcomes has been shown to be variable. For example, a similar result was measured in one study that used a HFD, (23), but a second research group measured decreased diversity in mice on a HFD or high fructose diet (47). In this study, we were further able to detect that many of these effects were dependent on obesity status. Obesity on the WD and HFD was associated with a significant decrease in Faith's PD and observed features. Relative to the lean-HFD, lean-WD mice had more richness measured by Faith's PD and observed features, but there was no analogous difference between the obese-HFD and obese-WD. This interaction between diet and obesity continued to appear in further analyses.

Using weighted and unweighted UniFrac distances, we detected statistical differences between all six groups based on pairwise comparisons. Upon further examination of the PCoA generated from weighted UniFrac distances, we found that the microbial communities of lean-HFD and lean-WD clustered more distinctly from each other compared to the obese-HFD and obese-WD, which was more variable and had greater overlap. This same pattern was measured in intestinal permeability. Lean-HFD and lean-WD had stronger barrier function relative to the lean-LFD, while there were no differences in barrier function among obese groups. Since lean mice on the HFD and WD had increased taxa richness relative to the lean mice on the LFD, these results

support the notion that higher alpha diversity is associated with better health outcomes. This was unexpected given the high saturated fat and sugar content of the HFD and WD. These results highlight the importance of obesity status on the diet-gut microbiota relationship and the potential impact on intestinal permeability.

There was much overlap of significantly enriched taxa between lean-HFD mice and lean-WD mice that may protect and/or strength the intestinal barrier. For example, *Clostridium* of the *Ruminococcaceae* family and some members of the *Lachnospiraceae* family can produce short chain fatty acids (SCFA) such as butyrate (49). Butyrate is a major energy source for colonocytes, and has been shown to strengthen the intestinal barrier by promoting tight junction assembly, reducing inflammation, and promoting mucus production (50–54). *Coprobacillus* has been previously shown to be enriched in lean mice (55) and is associated with consumption of fast-foods such as meats, French fries, mayonnaise and soft drinks, a phenotype similar to lean mice on obesogenic diets (56). Decreased *Coprobacillus* has been associated with damage to the intestinal barrier (57) and increased *Coprobacillus* has been associated with butyrate mediated restoration of the intestinal barrier (58). These results suggest that this bacterium may play an important role in improving the intestinal barrier under obesogenic diet conditions. Similar to other studies, obese mice on the HFD and WD had significantly worse barrier function compared to their lean counterparts (4,5,59). Obese mice on the HFD and WD were enriched in members of the *Coriobacteriaceae* and *Erysipelotrichaceae* families which have been previously reported to be enriched in obesity, involved in metabolic disease, and increased inflammation (60–63). Some members of *Coriobacteriaceae* are involved in bile acid metabolism which can negatively impact gut barrier function (62,64–66) and the *Erysipelotrichaceae* family has been shown to be enriched in colorectal cancer (CRC) patients (67) and in animal models of CRC (68). Together, this identifies potential microbes mediated by diet and obesity status that may be important in mediating intestinal permeability.

In this study, we have demonstrated that developing obesity or remaining lean on an obesogenic diet has significant effects on the gut microbiota and intestinal permeability. Despite high amounts of saturated fat and sugar, lean mice on the HFD or WD had significantly better barrier function relative to lean mice on the LFD. A limitation here is that only the *in vivo* measurement of intestinal permeability was significant. We did not detect any differences in MUC2 or ZO-1 expression, likely because the sample size was too small. Further validation of this

outcome through assessments such as serum lipopolysaccharide protein (LPS) is needed. In addition, permeability is only one marker of intestinal health. Analyzing other components in the colon such as pro and anti-inflammatory gene expression and fecal metabolites would enable us to better understand the possible mechanisms linking obesity, diet, gut microbiota, and colonic health. Our research demonstrates the need to better understand how specific dietary components can mediate the gut microbiota and various aspects of colonic health. This will improve our ability to effectively use diet in disease prevention.

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2.7 References

1. Ward ZJ, Long MW, Resch SC, Giles CM, Cradock AL, Gortmaker SL. Simulation of Growth Trajectories of Childhood Obesity into Adulthood. N Engl J Med. 2017 Nov 30;377(22):2145–53.

2. Ward ZJ, Bleich SN, Cradock AL, Barrett JL, Giles CM, Flax C, et al. Projected U.S. State-Level Prevalence of Adult Obesity and Severe Obesity. N Engl J Med. 2019 Dec 19;381(25):2440–50.

3. Centers for Disease Control and Prevention. Adult Obesity Causes & Consequences [Internet]. Division of Nutrition, Physical Activity, and Obesity, National Center for Chronic Disease Prevention and Health Promotion. Available from: https://www.cdc.gov/obesity/adult/causes.html

4. Cani PD, Bibiloni R, Knauf C, Waget A, Neyrinck AM, Delzenne NM, et al. Changes in Gut Microbiota Control Metabolic Endotoxemia-Induced Inflammation in High-Fat Diet–Induced Obesity and Diabetes in Mice. Diabetes. 2008 Jun 1;57(6):1470–81.

5. Cani PD, Possemiers S, Van de Wiele T, Guiot Y, Everard A, Rottier O, et al. Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability. Gut. 2009 Aug 1;58(8):1091–103.

6. de La Serre CB, Ellis CL, Lee J, Hartman AL, Rutledge JC, Raybould HE. Propensity to high-fat diet-induced obesity in rats is associated with changes in the gut microbiota and gut inflammation. Am J Physiol-Gastrointest Liver Physiol. 2010 Aug;299(2):G440–8.

7. Luck H, Tsai S, Chung J, Clemente-Casares X, Ghazarian M, Revelo XS, et al. Regulation of Obesity-Related Insulin Resistance with Gut Anti-inflammatory Agents. Cell Metab. 2015 Apr;21(4):527–42.

8. Duan M, Wang Y, Zhang Q, Zou R, Guo M, Zheng H. Characteristics of gut microbiota in people with obesity. Ling Z, editor. PLOS ONE. 2021 Aug 10;16(8):e0255446.

9. Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende DR, et al. Enterotypes of the human gut microbiome. Nature. 2011 May 12;473(7346):174–80.

10. De Filippo C, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, Massart S, et al. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proc Natl Acad Sci. 2010 Aug 17;107(33):14691–6.

11. Walker AW, Ince J, Duncan SH, Webster LM, Holtrop G, Ze X, et al. Dominant and diet-responsive groups of bacteria within the human colonic microbiota. ISME J. 2011 Feb;5(2):220–30.

12. Wu GD, Chen J, Hoffmann C, Bittinger K, Chen Y-Y, Keilbaugh SA, et al. Linking Long-Term Dietary Patterns with Gut Microbial Enterotypes. Science. 2011 Oct 7;334(6052):105–8.

13. Cotillard A, Kennedy SP, Kong LC, Prifti E, Pons N, Le Chatelier E, et al. Dietary intervention impact on gut microbial gene richness. Nature. 2013 Aug 29;500(7464):585–8.

14. Murphy EF, Cotter PD, Healy S, Marques TM, O'Sullivan O, Fouhy F, et al. Composition and energy harvesting capacity of the gut microbiota: relationship to diet, obesity and time in mouse models. Gut. 2010 Dec;59(12):1635–42.

15. David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, et al. Diet rapidly and reproducibly alters the human gut microbiome. Nature. 2014 Jan;505(7484):559–63.

16. Rothschild D, Weissbrod O, Barkan E, Kurilshikov A, Korem T, Zeevi D, et al. Environment dominates over host genetics in shaping human gut microbiota. Nature. 2018 Mar;555(7695):210–5.

17. Carmody RN, Gerber GK, Luevano JM, Gatti DM, Somes L, Svenson KL, et al. Diet dominates host genotype in shaping the murine gut microbiota. Cell Host Microbe. 2015 Jan 14;17(1):72–84.

18. Mozaffarian D, Hao T, Rimm EB, Willett WC, Hu FB. Changes in Diet and Lifestyle and Long-Term Weight Gain in Women and Men. N Engl J Med. 2011 Jun 23;364(25):2392–404.

19. Kopp W. How Western Diet And Lifestyle Drive The Pandemic Of Obesity And Civilization Diseases. Diabetes Metab Syndr Obes Targets Ther. 2019 Oct;Volume 12:2221–36.

20. Hall KD, Ayuketah A, Brychta R, Cai H, Cassimatis T, Chen KY, et al. Ultra-Processed Diets Cause Excess Calorie Intake and Weight Gain: An Inpatient Randomized Controlled Trial of Ad Libitum Food Intake. Cell Metab. 2019 Jul;30(1):67-77.e3.

21. Vandevijvere S, Chow CC, Hall KD, Umali E, Swinburn BA. Increased food energy supply as a major driver of the obesity epidemic: a global analysis. Bull World Health Organ. 2015 Jul 1;93(7):446–56.

22. Hildebrandt MA, Hoffmann C, Sherrill–Mix SA, Keilbaugh SA, Hamady M, Chen Y, et al. High-Fat Diet Determines the Composition of the Murine Gut Microbiome Independently of Obesity. Gastroenterology. 2009 Nov;137(5):1716-1724.e2.

23. Xiao L, Sonne SB, Feng Q, Chen N, Xia Z, Li X, et al. High-fat feeding rather than obesity drives taxonomical and functional changes in the gut microbiota in mice. Microbiome. 2017 Dec;5(1):43.

24. Bäckhed F, Ding H, Wang T, Hooper LV, Koh GY, Nagy A, et al. The gut microbiota as an environmental factor that regulates fat storage. Proc Natl Acad Sci U S A. 2004 Nov 2;101(44):15718–23.

25. Bäckhed F, Manchester JK, Semenkovich CF, Gordon JI. Mechanisms underlying the resistance to diet-induced obesity in germ-free mice. Proc Natl Acad Sci U S A. 2007 Jan 16;104(3):979–84.

26. Hooper LV, Stappenbeck TS, Hong CV, Gordon JI. Angiogenins: a new class of microbicidal proteins involved in innate immunity. Nat Immunol. 2003 Mar;4(3):269–73.

27. Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R. Recognition of Commensal Microflora by Toll-Like Receptors Is Required for Intestinal Homeostasis. Cell. 2004 Jul;118(2):229–41.

28. Mazmanian SK, Liu CH, Tzianabos AO, Kasper DL. An Immunomodulatory Molecule of Symbiotic Bacteria Directs Maturation of the Host Immune System. Cell. 2005 Jul;122(1):107–18.

29. Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly-Y M, et al. The Microbial Metabolites, Short-Chain Fatty Acids, Regulate Colonic T_{reg} Cell Homeostasis. Science. 2013 Aug 2;341(6145):569–73.

30. Atarashi K, Tanoue T, Oshima K, Suda W, Nagano Y, Nishikawa H, et al. Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. Nature. 2013 Aug 8;500(7461):232–6.

31. Martinez-Medina M, Denizot J, Dreux N, Robin F, Billard E, Bonnet R, et al. Western diet induces dysbiosis with increased *E coli* in CEABAC10 mice , alters host barrier function favouring AIEC colonisation. Gut. 2014 Jan;63(1):116–24.

32. Kim K-A, Gu W, Lee I-A, Joh E-H, Kim D-H. High Fat Diet-Induced Gut Microbiota Exacerbates Inflammation and Obesity in Mice via the TLR4 Signaling Pathway. Chamaillard M, editor. PLoS ONE. 2012 Oct 16;7(10):e47713.

33. Boulangé CL, Neves AL, Chilloux J, Nicholson JK, Dumas M-E. Impact of the gut microbiota on inflammation, obesity, and metabolic disease. Genome Med. 2016 Dec;8(1):42.

34. Lomba A, Milagro FI, García-Díaz DF, Marti A, Campión J, Martínez JA. Obesity induced by a pair-fed high fat sucrose diet: methylation and expression pattern of genes related to energy homeostasis. Lipids Health Dis. 2010 Dec;9(1):60.

35. Turnbaugh PJ, Ridaura VK, Faith JJ, Rey FE, Knight R, Gordon JI. The Effect of Diet on the Human Gut Microbiome: A Metagenomic Analysis in Humanized Gnotobiotic Mice. Sci Transl Med [Internet]. 2009 Nov 11 [cited 2022 Jan 1];1(6). Available from: https://www.science.org/doi/10.1126/scitranslmed.3000322

36. Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Appl Environ Microbiol. 2013 Sep;79(17):5112–20.

37. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol. 2019 Aug;37(8):852–7.

38. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods. 2016 Jul;13(7):581–3.

39. Callahan BJ, McMurdie PJ, Holmes SP. Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. ISME J. 2017 Dec;11(12):2639–43.

40. McDonald D, Price MN, Goodrich J, Nawrocki EP, DeSantis TZ, Probst A, et al. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. ISME J. 2012 Mar;6(3):610–8.

41. Lozupone C, Knight R. UniFrac: a New Phylogenetic Method for Comparing Microbial Communities. Appl Environ Microbiol. 2005 Dec;71(12):8228–35.

42. Benjamini Y, Hochberg Y. Controlling The False Discovery Rate - A Practical And Powerful Approach To Multiple Testing. Journal of the Royal Statistical Society Series B: Methodological. 1995 Nov;57:289–300.

43. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, et al. Metagenomic biomarker discovery and explanation. Genome Biol. 2011;12(6):R60.

44. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nat Methods. 2012 Jul;9(7):671–5.

45. R Core Team. R: A Language and Environment for Statistical Computing [Internet]. Vienna, Austria: R Foundation for Statistical Computing; 2021. Available from: https://www.R-project.org/

46. Murphy EA, Velazquez KT, Herbert KM. Influence of high-fat diet on gut microbiota: a driving force for chronic disease risk. Curr Opin Clin Nutr Metab Care. 2015 Sep;18(5):515–20.

47. Tan R, Dong H, Chen Z, Jin M, Yin J, Li H, et al. Intestinal Microbiota Mediates High-Fructose and High-Fat Diets to Induce Chronic Intestinal Inflammation. Front Cell Infect Microbiol. 2021 Jun 16;11:654074.

48. Kong C, Gao R, Yan X, Huang L, Qin H. Probiotics improve gut microbiota dysbiosis in obese mice fed a high-fat or high-sucrose diet. Nutrition. 2019 Apr;60:175–84.

49. Flint HJ, Scott KP, Duncan SH, Louis P, Forano E. Microbial degradation of complex carbohydrates in the gut. Gut Microbes. 2012 Aug;3(4):289–306.

50. Jiminez JA, Uwiera TC, Abbott DW, Uwiera RRE, Inglis GD. Butyrate Supplementation at High Concentrations Alters Enteric Bacterial Communities and Reduces Intestinal Inflammation in Mice Infected with Citrobacter rodentium. Suen G, editor. mSphere. 2017 Aug 30;2(4):e00243-17.

51. Roediger WE. Role of anaerobic bacteria in the metabolic welfare of the colonic mucosa in man. Gut. 1980 Sep 1;21(9):793–8.

52. Peng L, Li Z-R, Green RS, Holzman IR, Lin J. Butyrate Enhances the Intestinal Barrier by Facilitating Tight Junction Assembly via Activation of AMP-Activated Protein Kinase in Caco-2 Cell Monolayers. J Nutr. 2009 Sep 1;139(9):1619–25.

53. Wang RX, Lee JS, Campbell EL, Colgan SP. Microbiota-derived butyrate dynamically regulates intestinal homeostasis through regulation of actin-associated protein synaptopodin. Proc Natl Acad Sci. 2020 May 26;117(21):11648–57.

54. Nielsen DSG, Jensen BB, Theil PK, Nielsen TS, Knudsen KEB, Purup S. Effect of butyrate and fermentation products on epithelial integrity in a mucus-secreting human colon cell line. J Funct Foods. 2018 Jan;40:9–17.

55. Nishitsuji K, Xiao J, Nagatomo R, Umemoto H, Morimoto Y, Akatsu H, et al. Analysis of the gut microbiome and plasma short-chain fatty acid profiles in a spontaneous mouse model of metabolic syndrome. Sci Rep. 2017 Dec;7(1):15876.

56. Bolte LA, Vich Vila A, Imhann F, Collij V, Gacesa R, Peters V, et al. Long-term dietary patterns are associated with pro-inflammatory and anti-inflammatory features of the gut microbiome. Gut. 2021 Jul;70(7):1287–98.

57. Yu L, Yu Y, Yin R, Duan H, Qu D, Tian F, et al. Dose-dependent effects of lead induced gut injuries: An in vitro and in vivo study. Chemosphere. 2021 Mar;266:129130.

58. Ye J, Lv L, Wu W, Li Y, Shi D, Fang D, et al. Butyrate Protects Mice Against Methionine–Choline-Deficient Diet-Induced Non-alcoholic Steatohepatitis by Improving Gut Barrier Function, Attenuating Inflammation and Reducing Endotoxin Levels. Front Microbiol. 2018 Aug 21;9:1967.

59. Lam YY, Ha CWY, Campbell CR, Mitchell AJ, Dinudom A, Oscarsson J, et al. Increased Gut Permeability and Microbiota Change Associate with Mesenteric Fat Inflammation and Metabolic Dysfunction in Diet-Induced Obese Mice. Zhang RR, editor. PLoS ONE. 2012 Mar 23;7(3):e34233.

60. Qin Y, Roberts JD, Grimm SA, Lih FB, Deterding LJ, Li R, et al. An obesity-associated gut microbiome reprograms the intestinal epigenome and leads to altered colonic gene expression. Genome Biol. 2018 Dec;19(1):7.

61. Zhang H, DiBaise JK, Zuccolo A, Kudrna D, Braidotti M, Yu Y, et al. Human gut microbiota in obesity and after gastric bypass. Proc Natl Acad Sci. 2009 Feb 17;106(7):2365–70.

62. Clavel T, Lepage P, Charrier C. The Family Coriobacteriaceae. In: Rosenberg E, DeLong EF, Lory S, Stackebrandt E, Thompson F, editors. The Prokaryotes [Internet]. Berlin, Heidelberg: Springer Berlin Heidelberg; 2014 [cited 2022 Mar 10]. p. 201–38. Available from: http://link.springer.com/10.1007/978-3-642-30138-4_343

63. Dinh DM, Volpe GE, Duffalo C, Bhalchandra S, Tai AK, Kane AV, et al. Intestinal Microbiota, Microbial Translocation, and Systemic Inflammation in Chronic HIV Infection. J Infect Dis. 2015 Jan 1;211(1):19–27.

64. Wegner K, Just S, Gau L, Mueller H, Gérard P, Lepage P, et al. Rapid analysis of bile acids in different biological matrices using LC-ESI-MS/MS for the investigation of bile acid transformation by mammalian gut bacteria. Anal Bioanal Chem. 2017 Feb;409(5):1231–45.

65. Stenman LK, Holma R, Eggert A, Korpela R. A novel mechanism for gut barrier dysfunction by dietary fat: epithelial disruption by hydrophobic bile acids. Am J Physiol-Gastrointest Liver Physiol. 2013 Feb 1;304(3):G227–34.

66. Turnbaugh PJ. Fat, bile and gut microbes. Nature. 2012 Jul;487(7405):47–8.

67. Chen W, Liu F, Ling Z, Tong X, Xiang C. Human Intestinal Lumen and Mucosa-Associated Microbiota in Patients with Colorectal Cancer. Moschetta A, editor. PLoS ONE. 2012 Jun 28;7(6):e39743.

68. Zhu Q, Jin Z, Wu W, Gao R, Guo B, Gao Z, et al. Analysis of the Intestinal Lumen Microbiota in an Animal Model of Colorectal Cancer. Hold GL, editor. PLoS ONE. 2014 Mar 6;9(3):e90849.

CHAPTER 3. OBESOGENIC DIETS INDEPENDENT FROM THE DEVELOPMENT OF OBESITY INCREASE CRC RISK BY MODULATING GUT MICROBIOTA

3.1 Abstract

Obesity is associated with increased risk for the development of colorectal rectal cancer (CRC). Although there are a variety of components that contribute to this link, obesity-associated gut microbiota may be an important factor mediating this disease. To isolate the role of obesity-associated gut microbiota in CRC, we transplanted feces from donor mice that developed obesity or remained lean on a high fat diet (HFD) or western diet (WD) into recipient mice that were then treated with azoxymethane (AOM) to induce CRC development. We hypothesized that the gut microbiota from obese mice on obesogenic diets would promote CRC independent from the development of obesity. Our results showed that relative to LFD-recipients, HFD-recipients had worse tumor incidence and number, and WD-recipients displayed more cellular signs of a tumorigenic environment. In addition, WD-recipients had increased expression of the immune cell inhibitor receptor SIRPα, relative to LFD-recipients. Collectively, these results suggest that gut microbiota of WD-recipients still promote CRC but have components that attenuate its development. Differences in taxa richness, abundance, and community composition in fecal microbiota did not remain the same before and after tumor development. Significantly enriched taxa present before tumor development in HFD and WD recipients such as *Sutterella, Dorea, Bacteroidetes* and *Enterococcus* and those enriched after tumor development such as *Blautia, Erysipelotrichaceae*, and *Anaeroplasma* may be influencing the progression of CRC in HFD and WD recipients. In sum, our results demonstrate that gut microbiota shaped by obesogenic diets significantly promote CRC development. This highlights the importance of assessing the role of different diets in the gut microbiota-CRC relationship, as these effects can promote and/or prevent CRC development.

3.2 Introduction

Colorectal cancer (CRC) is the third most common cancer and second leading cause of cancer deaths in the United States (1). While overall incidence has been steadily decreasing, when

stratified by age, researchers have measured an increase in CRC cases in younger populations (2–4). A factor that may be contributing to this trend is obesity, which is steadily rising in adolescents and adults (5,6). Obesity has been consistently associated with increased risk for the development of CRC (1,7,8). In animal studies, tumor growth and preneoplastic lesion development were positively associated with diet-induced obesity and negatively associated with calorie restriction and weight loss (9–13). One mechanism linking obesity and CRC is inflammation. Chronic low-grade inflammation commonly present in obese patients stems largely from excess adipose tissue. Tissue resident macrophages and adipocytes can produce and secrete pro-inflammatory cytokines, chemokines, hormones, and growth factors that generate an ideal environment for CRC development (14–17).

Considering the location of CRC, another component that influences inflammation-promoting tumorigenesis is the gut microbiota. The colon harbors trillions of commensal bacteria that have been shown to play key roles in preventing intestinal permeability and promoting anti-inflammatory immune cell phenotypes (18). Changes to the gut microbiota associated with a diseased state, termed dysbiosis, can disturb colonic homeostasis to promote disease progression (19,20). It has been linked to obesity (21–24) and to CRC risk (19,25–30). By modulating the gut microbiota through antibiotics, probiotics, and fecal microbial transplants (FMTs), researchers have demonstrated that intestinal microorganisms can significantly influence colonic health and the risk for developing CRC (31–38).

The obese gut microbiota is associated with mediating an inflammatory, pro-cancer colonic environment, but few studies have adequately isolated its role in CRC development independent from the development of obesity. These research groups found that diets high in fat promote CRC development by modulating the gut microbiota. However, there were a few limitations. These studies used a small intestine model of cancer, mice that were resistant to diet-induced obesity, and/or a control group that did not adequately assess the effect of obesity-associated gut microbiota on CRC development (39–41). Most human CRC cases occur in the colon and rectum, and the microbiota of the small intestine and large intestine are vastly different in composition and community structure (42,43). In addition, these studies relied on only a high fat diet to model an obese gut microbiota. Since diet drives obesity and is the primary source of substrates for intestinal microbes, it is critical to assess and compare the role of other obesogenic diets in mediating the gut microbiota-CRC relationship (44).

To this end, we used fecal microbial transplantation (FMT) to determine (a) if obesity-associated gut microbiota influences the development of CRC, independent from the development of obesity and (b) if the development of CRC differs based on the obesogenic diet consumed by FMT donors. We hypothesized that microbiota from obese mice on obesogenic diets would promote CRC independent from the development of obesity, and while microbiota from lean mice on obesogenic diets would not promote tumor development, it would be associated with worse colonic health relative to a control.

3.3 Methods

3.3.1 Study design and animal husbandry

Donor mice (from Chapter 2): Six-week-old male wildtype C57BL/6J mice (n=20/group) and *ob/ob* mice on a C57BL/6J background (n=20) were purchased from Jackson Laboratories and fed a low-fat diet (LFD, 10 kcal% fat, #D12450J, Research Diets, Inc., New Brunswick, NJ, USA) *ad libitum* for one week of acclimatization. *Ob/ob* mice express a mutation in the leptin gene that induces hyperphagia. This group was maintained on the LFD throughout the study and was included to generate an obese-LFD group. C57BL/6J mice were then randomized into 5 diet groups (see table 2.1 for diet composition): (i) LFD, (ii) high-fat diet (HFD, 60% of kcals from fat), (iii) Western diet (WD, 40% kcals from fat, 30% kcals from sucrose), (iv) pair-fed HFD (lean-HFD), and (v) pair-fed WD (lean-WD). Pair-fed mice received a daily ration of HFD or WD that was isocaloric to the number of kilocalories (kcals) consumed by the lean-LFD control group a week prior. Previous research indicated that pair-fed mice on obesogenic diets still gain more weight relative to a LFD control (45). We monitored and recorded body weight twice a week and adjusted the number of kcals consumed so that lean-HFD and lean-WD had comparable weight to lean-LFD. Mice were pair-fed starting at week 6. After 15 weeks on these dietary interventions, mice were euthanized with CO_2 and cervical dislocation. At euthanasia, cecal and fecal material were processed in an anaerobic chamber (Plas-Labs 855-AC, Lansing MI) and suspended in 1.875 mL anaerobic phosphate buffered saline and 1.125 mL of 40% glycerol in a 15 mL sterile conical tube. Fecal and cecal material was homogenized via pestle and vortex then stored as 300 uL aliquots in the -80C freezer until FMT.

Recipient mice: Nine-week-old male wildtype FVB/N (20/group) were acclimated on the LFD *ad libitum* for 1-week. Mice then received a 7-day treatment with antibiotic cocktail via daily oral gavage (10 mg/kg ampicillin, 5 mg/kg vancomycin, 5 mg/kg neomycin, 10 mg/kg metronidazole; 10 ml/kg) to knock-down the gut microbiota. They were then randomized to receive an FMT via oral gavage from 1 of the 6 donor groups, with each donor mouse having a corresponding recipient mouse (n=20/grp). One week after the FMT, all mice received five weekly injections of azoxymethane (AOM, 10 mg/kg body weight/week i.p.). All recipient mice continued the LFD *ad libitum* and received an FMT every 3 weeks to maintain colonization until euthanasia 15 weeks after the final AOM injection. Study design illustrated in Figure 3.1. Fresh fecal pellets were collected at baseline (after LFD acclimatization), one week after the first FMT (post-donor colonization/pre-tumor) and one week prior to euthanasia (post-tumor) and stored in the -80°C freezer until processing for 16S rRNA gene sequencing. At euthanasia, tumors in the colon were counted and measured with a caliper. The colon was resected and rolled from distal to proximal end, fixed in 10% neutral buffered formalin for 48 hours, then paraffin embedded for histological analysis. While C57BL/6 mice were used as donor mice, they cannot be used as recipient mice because they are less sensitive to AOM-induced tumorigenesis. Concurrently, FVB/N will develop on average 5 colonic tumors after 5 weekly injections of AOM but are resistant to diet-induced obesity (31).

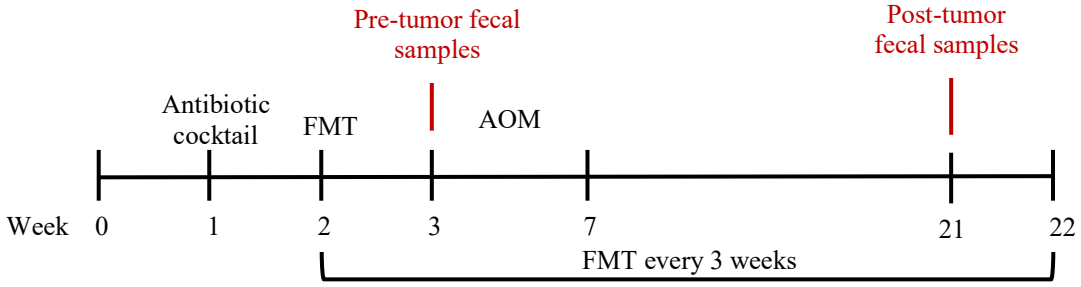


Figure 3.1 Experimental design for fecal microbial transplants (FMTs)

Nine-week-old male FVB/N mice were acclimated on a low-fat diet for 1 week, then orally gavaged with an antibiotic cocktail once a day for 7 days. Mice were then recolonized with donor gut microbiota. One week after colonization, colorectal cancer (CRC) was induced through 5 weekly intraperitoneal injections of azoxymethane (AOM). FMTs were continued every three weeks until euthanasia, 15 weeks after last AOM injection. Pre-tumor samples for 16S rRNA gene sequencing were collected one week after the first FMT and prior to the first AOM injection. Post-tumor fecal samples for 16S rRNA gene sequencing were collected one week before euthanasia. n= 18-20/group

3.3.2 Fecal microbial transplant (FMT)

On the day of FMT, inoculants were thawed to room temperature and 200 uL was administered to each mice via oral gavage. FMTs were performed every 3 weeks to maintain donor community in recipients.

3.3.3 Gut microbiota analysis using 16S rRNA gene sequencing

Genomic DNA was extracted from fecal contents using a bead-beating protocol (46). Briefly, ~ 25 mg of fecal pellet sample was suspended in a solution containing 500 µl of extraction buffer [200 mM Tris:HCl (pH 8.0), 200 mM NaCl, 20 mM EDTA], 210 µl of 20% SDS, 500 µl of Invitrogen™ UltraPure™ phenol:chloroform:isoamyl alcohol (cat# 15593049, Waltham, MA), and 500 µl of 0.1-mm diameter zirconia/silica beads. Samples were mechanically disrupted for 3 min at room temperature using a bead beater at 3400 RPM (Mini-beadbeater 96, BioSpec, Barlesville, OK), followed by centrifugation. The aqueous phase was recovered using 60 µl of 3 M NaAcetate and isopropanol precipitation. QIAquick 96-well PCR Purification Kit (Qiagen, cat# 28181, Germantown, MD) was used to remove contaminants. Isolated DNA was eluted in 10 mM TE (pH 8.0) buffer and was stored at −20 °C until further use.

Amplification of the 16S rRNA gene targeting the V4 region was generated from extracted genomic DNA by PCR using unique 8-bp barcodes on the forward and reverse primers and fused with Illumina sequencing adapters (47). Each sample was amplified in duplicate in a reaction volume of 25 uL using KAPA HiFi HotStart DNA polymerase (KAPA Biosystems, cat# KK2602, Wilmington, MA), 10 µM of each primer, and 12.5 ng of genomic DNA. PCR was carried out under the following conditions: initial denaturation for 3 min at 95°C, followed by 25 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 55°C and elongation for 30 s at 72°C, and a final elongation step for 5 min at 72°C. PCR products were purified with Sera-Mag Select beads using left side size selection as per the manufacturer's protocol (Cytiva, cat# 29343052, Shrewsbury, MA,). Equimolar amounts of each sample were pooled and then sequenced using the Illumina MiSeq platform with V2 chemistry (2 × 250 bp) at the Bindley Core for Genomics at Purdue University.

Sequences were processed using a QIIME 2 pipeline (48). Demultiplexed 250 bases paired-end sequences were imported using Casava 1.8 format and denoised using DADA2 (49,50) to

obtain an amplicon sequence variant (ASV) table. ASVs present in less than 11 samples were discarded for pre-tumor timepoint, and ASVs present in less than 22 samples were discarded for post-tumor data. Greengenes reference sequences (clustered at 99% similarity) were used to train a naïve Bayes taxonomy classifier to further annotate ASVs taxonomically (51,52). Alpha- and beta-diversity were measured using an even sampling depth of 9,419 sequences per sample on pre-tumor gut microbiota and 5,908 sequences per sample on post-tumor gut microbiota. Linear regression was used to assess differences among groups for alpha diversity metrics (Shannon diversity index (53), Faith's phylogenetic diversity (PD) (54), observed taxonomic units (OTUs), and Pielou's Evenness (55)). Beta-diversity was measured using unweighted (56) and weighted UniFrac distances (57) and visualized using principal coordinates analysis (PCoA). Differences in weighted UniFrac distances between microbiota communities were tested by pairwise PERMANOVA using QIIME2 beta-group-significance command with the -p-pairwise parameter. P-values corrected for multiple comparisons using Benjamini-Hochberg (58) is denoted by q-values. Linear discriminant analysis (LDA) effect size (LEfSe Galaxy Version 1.0) was performed to identify differentially abundant bacteria using default p value ($\alpha = 0.05$), LDA score of 2.0, and one against all for multi-class analysis strategy (59).

3.3.4 Colon RNA processing and analysis

Using a microtome, 4 slices (10 micron/slice) of paraffin embedded colon samples were put into 1.5 mL Eppendorf tubes and stored in the -80°C freezer. Prior to RNA extraction, samples were deparaffinized using deparaffinization solution {Qiagen, Germantown, MD, 19093) and the manufacturer's protocol. RNA was extracted using Norgen Biotek Corp's FFPE RNA Purification Kit (Ontario, Canada, cat# 25300) per manufacturer's protocol. Sample quality was checked using the Agilent Bioanalyzer 2100 and all samples were greater than 300 nucleotides. Sample concentration was determined via ThermoScientific™ NanoDrop™ One Microvolume UV-Vis Spectrophotometer (Waltham, MA, cat# 13-400-519) and diluted to 40 ng/μL. Samples were further processed to be run on the nCounter Analysis system at Indiana University-Purdue University Indianapolis (IUPUI) according to manufacturer's protocol. In brief, samples are first incubated and hybridized with target probes that are tagged with a unique florescent color-coded barcode. These hybridized probes are then bound to a cartridge and immobilized, and images are digitally captured by epifluorescence microscopy. Gene expression is then assessed by counting

barcodes captured by these images. NanoString nCounter PanCancer^{TM} IO 360 panel of 770 genes was used. (Seattle, WA, cat# 115000260).

Data was analyzed using nSolver 4.0. This program performs a quality check and performs technical and biological normalization by using a positive control and a set of housekeeping genes, respectively. For background correction we used background thresholding based on the geometric mean of negative controls. For normalization, we used the arithmetic mean of the geometric means of all housekeeping genes. Normalized data was used for all analyses. T-tests were performed on log2-transformed count data, and Benjamini-Hochberg correction was used to control for multiple comparisons (α=0.05). Fold change calculations are on log2 scale.

3.3.5 Histological assessment of colon-tumor

Colon sections 4 μm thick were stained with hematoxylin and eosin according to standard methods by the Histology Research Laboratory at Purdue University. Microscopic examination was performed by a blinded board-certified veterinary pathologist and the interpretation was based on standard histopathological morphology. To determine the extent of mouse colonic lesions, a semi-quantitative method was used to assess the amount of inflammatory infiltrate and distribution, epithelial necrosis of non-neoplastic mucosa, crypt distortion, and epithelial changes related to neoplasia using a histomorphological scale (Table 3.1)

Table 3.1 Histomorphological scale used to assess inflammation, epithelial necrosis, crypt architectural distortion and tumor grade in colon

Criterion	Score
Extent of inflammation	3= diffuse
	2= multifocal
	1 = focal/locally extensive
	0 = none
Severity of Inflammation	3= marked
	2= moderate
	1 = mild
	0 = none
Epithelial necrosis	3= 50% or greater of the cells
	2= 10%-50%
	1 = <10%
	0 = 0%
Crypt architectural distortion	3= 50% or greater of the crypts in the mucosa
	2= 10%-50%
	1 = <10%
	0 = Normal
Tumor grade	3= adenocarcinoma
	2= adenoma
	1 = mucosal hyperplasia
	0 = Normal

3.3.6 Statistical Analysis

All statistical analysis was performed using R version 4.1.2 (2021-11-01) (60) or QIIME2 (2021.4) (48). Two parameters, donor obesity status (obese or lean) and donor diet (LFD, HFD, WD), were used in each model. Tumor incidence was assessed using logistic regression; tumor number was assessed using negative binomial regression, and tumor burden was assessed using gamma generalized linear model (GLM). For histological scoring, we applied ordinal logistic regression,

logistic regression, or Firth's bias corrected logistic regression using ordinal and logistf R packages (61,62).

Choice of sample size: A previous unpublished study done by our lab showed that 20 mice per group was sufficient to detect the effect of a drug and/or weight loss on colon tumor incidence, number, and burden with an α=0.05. In this study we measure these same outcomes but consider the effect of gut microbiota collected from lean and obese mice on specific diets. Assuming that the treatment effects in this study would be approximately the same size or even slightly smaller than those in the previous study would justify a sample size of 20 per group. Other studies using fecal transplant treatments also indicate that roughly 20 or fewer mice per group can provide sufficient power for detecting effects on outcomes related to those studied here (39–41). Another approach to aid the determination of an appropriate sample size is to use a formal power calculation based on the statistical models that will be used to analyze the data. However, the nature of the outcomes we are measuring necessitate the use of generalized linear models or non-parametric procedures, for which such calculations are inherently difficult and typically rely on simulation-based methods (63). In this approach, one must similarly make speculations about the potential effect size of the treatments, which for a GLM comes down to specifying the linear coefficients without having seen any data, which is inherently difficult. Another weakness of such a priori power calculations is that they assume that the models are well-specified, which is unrealistic in practice. As such, the sample sizes indicated by this approach should only be interpreted as an optimistic estimate. As an example, we use the simulation-based method implemented in the R package 'skpr' to estimate the power of the hypothesis tests for the coefficients in a logistic regression of tumor incidence on donor diet and donor obesity status (ignoring possible interaction terms). With 20 mice per group and under the assumption of an odds ratio of 2 for developing CRC in the HFD and WD recipients relative to the LFD control recipients (keeping obesity status fixed), and an odds ratio of 2 for obese recipients relative to lean recipients (keeping the diet fixed), the power of the associated hypothesis tests is greater than 85%, indicating that 20 mice per group would be sufficient.

Significance level: The use (and misuse) of p-values is a highly contentious issue in contemporary academic writing. Here, we follow the approach of the p-value's inventor, Ronald Fisher, who

when he described it in the 1920s never intended its evaluation against a pre-specified significance level to be a definitive test. Its purpose was to be used as an informal way to judge whether the evidence collected was significant enough to warrant further study (64). When choosing a reasonable significance level, it is valuable to discuss the potential losses associated with making either a false positive or a false negative conclusion. In the context of this study, the consequence of a false positive is to encourage further investigation into the topics investigated here, which in the worst-case scenario requires writing a grant and a grad student's hard labor. A false negative is arguably more detrimental to furthering the field as may discourage investigation into a particular line of study such as that of the role of diet and the gut microbiota in CRC development. As such, a p-value smaller than 0.1 is considered small enough to justify further investigation.

3.4 Results

Mice were weighed weekly. As expected, all mice gained weight but there were no significant differences in body weight among the six groups throughout our experimental period (Fig 3.2)

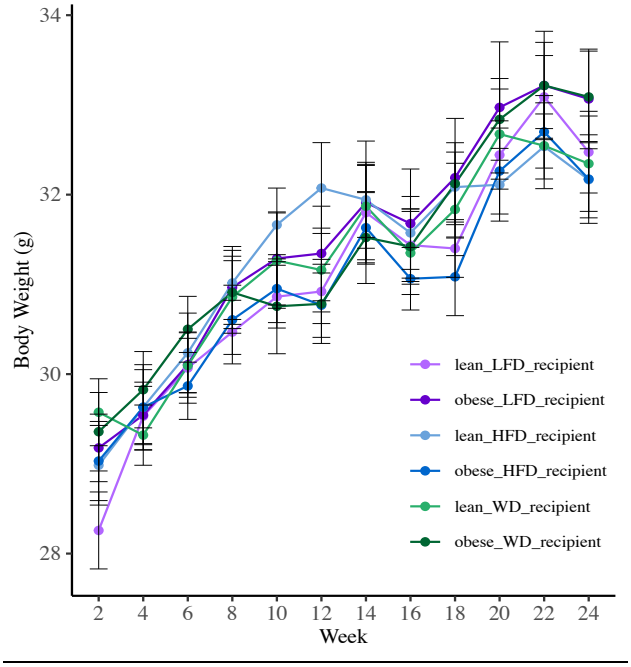


Figure 3.2 Body weight over time

Body weight was tracked weekly in FVB/N male mice receiving fecal microbial transplants (FMTs) from lean or obese donors that consumed a low-fat diet (LFD), high fat diet (HFD), or western diet (WD). Results expressed as mean +/- SE. Significance was calculated using 2-way ANOVA followed by Tukey HSD with α=0.05. n= 18-20/group

3.4.1 Obesity-associated gut microbiota promotes CRC independent from the development of obesity

By performing FMTs, we isolated the effect of obesity-associated gut microbiota on CRC development. Tumor incidence (Fig 3.3A), tumor number (Fig 3.3B), and tumor burden (Fig 3.3C) were not influenced based on donor obesity status. On the other hand, there was evidence that donor-diet was important in mediating these outcomes. To illustrate donor diet-based effects, we graphed tumor outcomes by combining lean and obese donor mice for each diet. HFD-recipient mice had higher tumor incidence compared to LFD-recipients ($p=0.065$) and WD-recipients ($p=0.077$). HFD-recipients had higher tumor number relative to LFD-recipients ($p=0.063$). We did not detect significant differences in tumor burden.

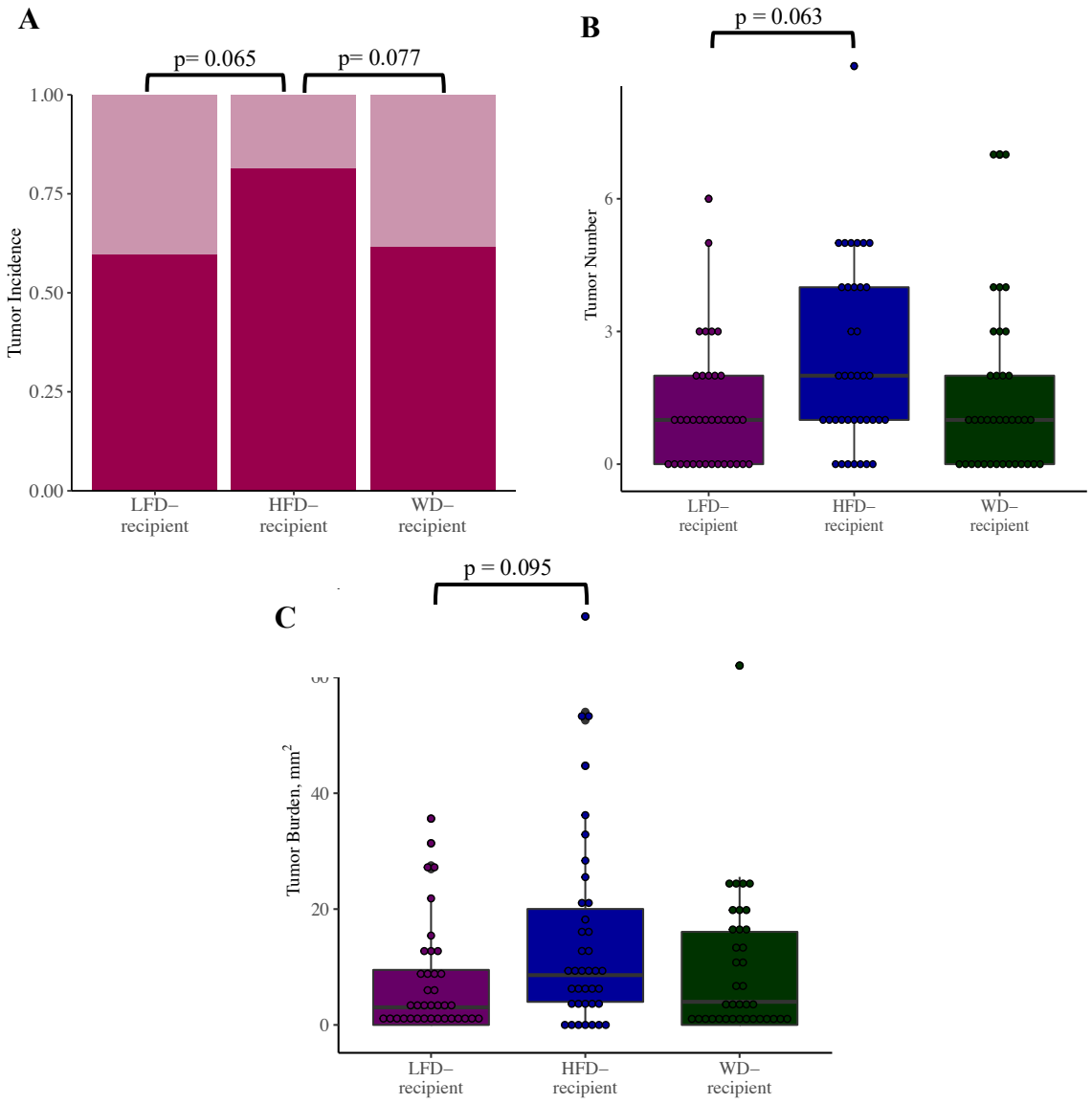


Figure 3.3 Macroscopic tumor outcomes

Colorectal cancer (CRC) was induced using 5 weekly injections of azoxymethane (AOM) in male FVB/N mice that had received an FMT from a donor mice that developed obesity or remained lean on a low-fat diet (LFD), high fat diet (HFD) or western diet (WD). Fifteen weeks after the last AOM injection, mice were euthanasia and (A) tumor incidence (B) tumor number and (C) tumor burden were assessed. For tumor incidence (A), dark pink represents proportion of mice that developed a tumor and light pink represents proportion of mice that developed no tumors, n = 36-40/group. Results were analyzed using logistic regression for tumor incidence, negative binomial regression for tumor number and gamma GLM for tumor burden. P-values are denoted on graphs

3.4.2 Gut microbiota from HFD and WD donors influence tumor histology

Histological outcomes of colon-tumor tissue were scored using ordered categories (0, 1, 2, 3). See Table 3.1 for full scoring criteria. Given this data structure, ordinal logistic regression is typically an appropriate model. However, we observed separation in inflammation extent, inflammation severity, and crypt defects. To address this, a binary variable was created in which low indicates a score smaller than or equal to one and high indicates a score larger than or equal to 2. This does not resolve separation, but it allows us to apply Firth's bias corrected logistic regression. Donor diet was a significant predictor for inflammation extent, inflammation severity, and crypt defects while donor obesity status was not. To visualize these results, outcomes were graphed based on proportion of high and low scores and by donor diet (Fig 3.4A-C). HFD-recipients and WD-recipients were significantly more likely to have higher inflammation extent scores relative to LFD-recipients (Fig 3.4A). WD-recipients were also significantly more likely to have higher inflammation severity relative to both LFD-recipients and HFD-recipients (Fig 3.4B). WD-recipients were statistically less likely to have more crypt defects relative to both LFD-recipients and HFD-recipients (Fig 3.4C). Since colon-tumor samples scored either a 0 or 1 for epithelial necrosis, logistic regression was used. WD-recipients were more likely to have higher ($p=0.083$) epithelial necrosis scores compared to LFD-recipients (Fig 3.4D). Ordinal logistic regression was used to assess tumor grade. In this analysis, we detected an interaction ($0.1>p>0.05$) between donor obesity status and donor diet. Only the obese-HFD-recipients were more likely to have lower tumor grade compared to lean-HFD-recipients ($p=0.016$). There were no differences in tumor grade between lean and obese LFD-recipients and lean and obese-HFD-recipients. Lean-HFD-recipients were more likely ($p=0.08$) to have high tumor grade compared to lean-LFD-recipients.

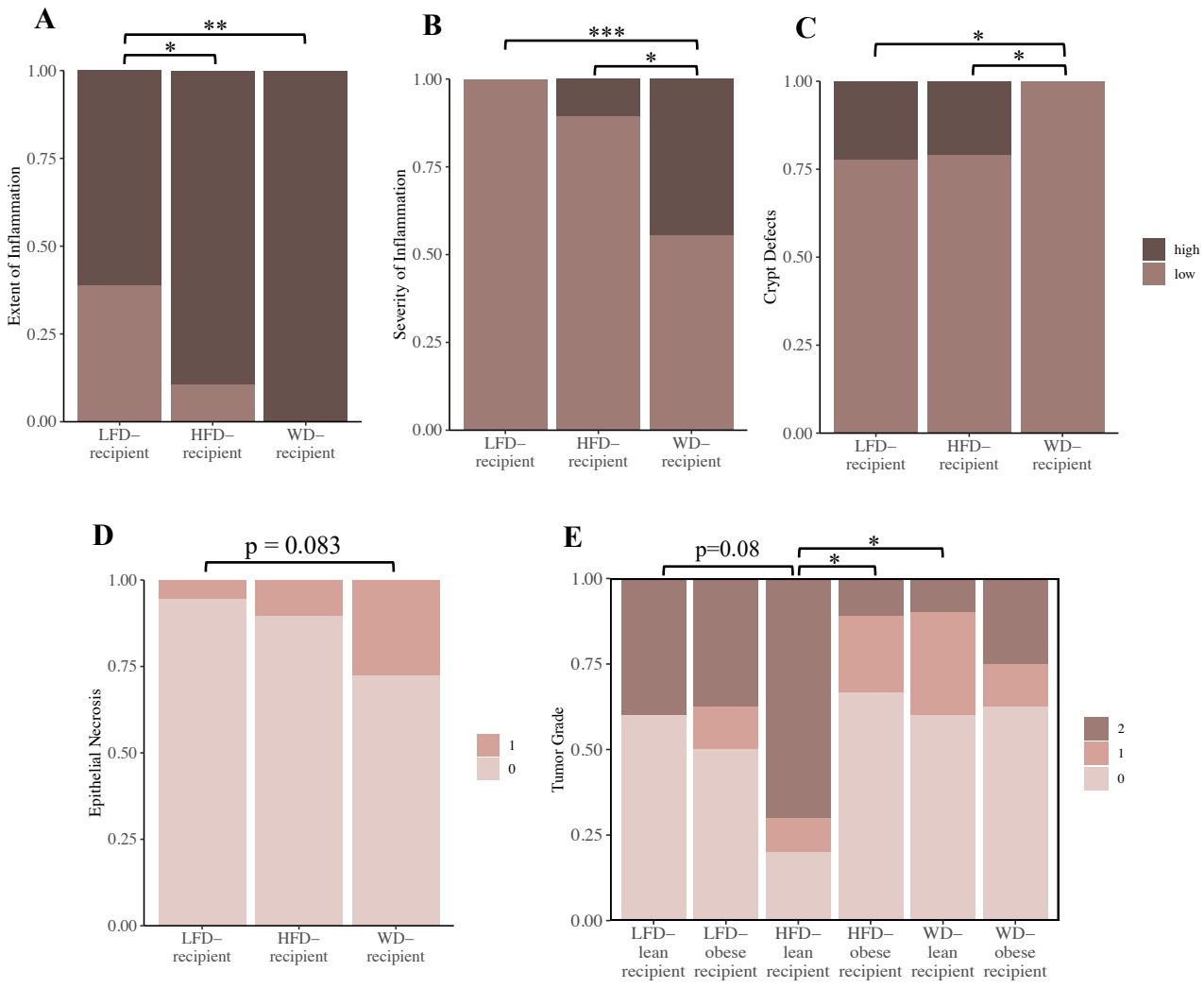


Figure 3.4 Histological assessment of colon-tumor tissue

A certified histopathologist scored colon specimens collected from male FVB/N mice that received a fecal microbial transplant (FMT) from lean and obese donors on a low-fat diet (LFD), high fat diet (HFD), or western diet (WD). (A) extent of inflammation (B) severity of inflammation (C) crypt defects and (D) epithelial necrosis were graphed by combining recipient data based only on donor diet, n=18-20/group. (E) Tumor grade was graphed based on obesity and diet status of donor mice, n=8-10/group. (A-C), dark brown is proportion of mice that had high histological scores (≥2) and medium brown is proportion of mice that had low histological scores (≤1). (D-E), colors represent histological categories in which higher numbers reflect worse outcomes as described in Table 3.1. Results were analyzed using Firth's bias corrected logistic regression for inflammation extent, severity, and crypt defects, logistic regression for epithelial necrosis, or ordinal logistic regression for tumor grade. * p< 0.05, ** p<0.01, *** p< 0.001.

3.4.3 Alpha diversity of fecal samples collected prior to tumor development is influenced by donor diet and donor obesity status

The richness and abundance of microbiota from fecal samples collected pre-tumor development were assessed. There was a significant interaction between donor diet and donor obesity status in Shannon entropy (Fig 3.5A), observed features (Fig 3.5B), Pielou's evenness (Fig 3.5C) and Faith's PD (Fig 3.5D). In all four of these measurements, obese-WD-recipients had significantly lower values compared to lean-WD-recipients, and obese-HFD-recipients had comparable scores to lean-HFD-recipients. Interestingly, obese-LFD-recipients had the opposite effect, these mice had higher Shannon entropy compared to the lean-LFD-recipients. There was a significant donor diet effect in Pielou's evenness in that the LFD-recipients had higher scores relative to the WD-recipients, regardless of donor obesity status.

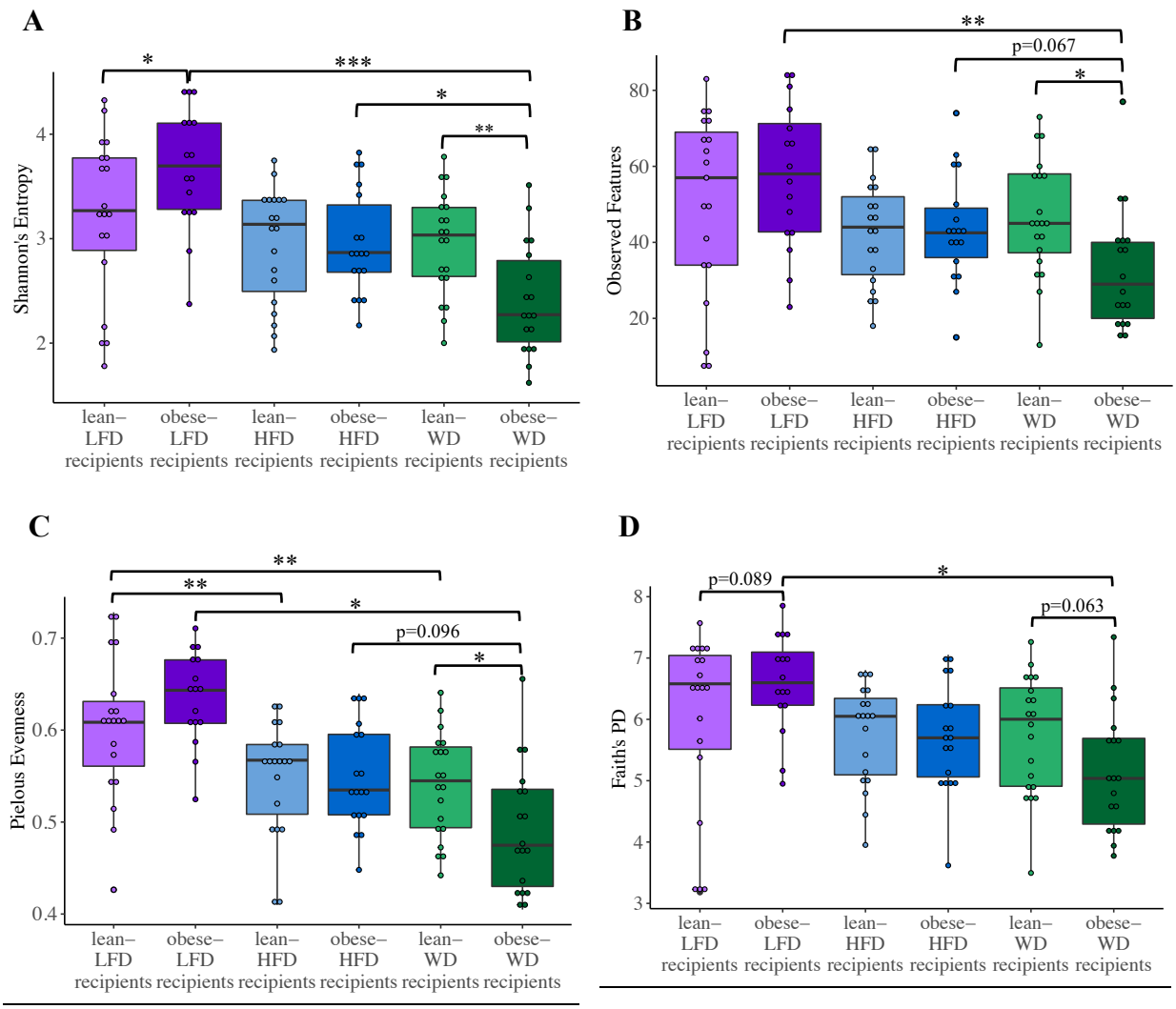


Figure 3.5 Alpha diversity of the fecal microbiota collected after donor colonization and before tumor development

Fecal samples were collected from male FVB/N recipient mice one week after receiving a fecal microbial transplant (FMT) from lean and obese donor mice that were on a low-fat diet (LFD), high fat diet (HFD) or western diet (WD). Data from 16S rRNA sequencing was used to assess alpha diversity using various metrics (A) Shannon's Entropy (B) observed features (c) Pielou's Evenness (D) Faith's PD. Six groups are colored by diet (LFD=purple, HFD=blue, WD=green), and obesity status, (light shade=lean, dark shade=obese), n=18-20/group. Data analyzed using linear regression * p< 0.05, **p <0.01

3.4.4 Beta diversity of fecal samples collected prior to tumor development is influenced by donor diet and donor obesity status

Differences in gut microbial community among the six groups prior to tumor development, were calculated using unweighted and weighted UniFrac distances and visualized with principal

coordinates analysis (PCoA). In the PCoA plot, a point represents a summary of the composition of the gut microbiota for one sample, incorporating phylogenetic relationship among microbes for uweighted UniFrac and also incorporating relative abundance for weighted UniFrac. The greater the distance between two points indicates the greater the dissimilarity between the two gut microbiotas. Using both unweighted and weighted UniFrac distances, we did not detect a significant main effect of donor obesity status (data not shown), but by donor-diet, LFD-recipients were significantly different from both HFD and WD-recipients, and HFD-recipients were significantly different than WD-recipients (Fig 3.6A). To determine if there were any interactions, pairwise comparisons were done among all six groups using weighted UniFrac distances (data not shown). Lean- and obese-LFD-recipients were different from each other (q=0.016), and lean- and obese-WD-recipients were different from each other (q=0.028), but there was no difference between lean-and obese-HFD recipients. Performing the same pairwise comparisons using unweighted UniFrac distances, we measured significant differences in pairwise comparisons among all six groups (data not shown). These results suggest that accounting for relative abundance increases the similarity between lean-and obese-HFD recipients' gut microbiota.

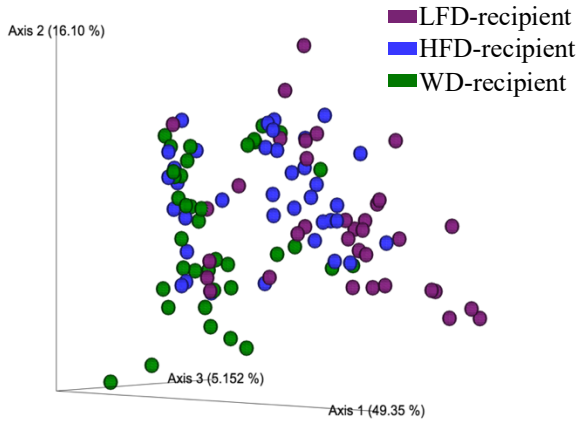


Figure 3.6 Beta diversity of the fecal microbiota collected prior to tumor development

Fecal samples were collected from male FVB/N recipient mice one week after receiving a fecal microbial transplant (FMT) from lean and obese donor mice that were on a low-fat diet (LFD), high fat diet (HFD) or western diet (WD). 16S rRNA sequencing data was used to determine beta-diversity and weighted UniFrac distances were used for principal coordinates analysis (PCoA) to visualize gut microbiota compositions of recipient communities, colored by donor diet, n=36-40/group. Analysis was done using PERMANOVA followed by pair-wise testing.

3.4.5 Significantly enriched taxa in fecal samples collected prior to tumor development

To determine if donor obesity status influenced taxa enrichment, we measured significantly enriched bacteria between lean-LFD-recipients and obese-LFD-recipients (Fig 3.7B), between lean-HFD-recipients and obese-HFD-recipients (Fig 3.7C), and between lean-WD-recipients and obese-WD-recipients (Fig 3.7D). Relative abundance was visualized by phyla (Fig 3.7A) and we found that Actinobacteria was significantly enriched in obese-LFD-recipients relative to lean-LFD-recipients; Actinobacteria and Tenericutes were significantly enriched in obese-HFD-recipients relative to lean-HFD-recipients. Between lean and obese WD recipients, Verrucomicrobia was significantly enriched in obese-WD, and Actinobacteria was significantly enriched in lean-WD-recipients. We also assessed differential abundance on a lower taxonomic level. Between lean and obese-LFD recipients, *Bacteroides* was enriched in lean-LFD-recipients while *Clostridium, Allobaculum, Erysipelotrichaceae, Lactobacillus,* and *S24-7* were enriched in obese-LFD-recipients. There was also only one significantly enriched taxon in the lean-HFD-recipients, *Clostridales*, while the obese-HFD-recipient was significantly enriched in *Anaeroplasma*, *Peptostreptococcaceae, Clostridium, Blautia, Christensenellaceae, Coriobacteriaceae* and *Coriobacteriales*. Obese-WD-recipients were enriched in only one genus, *Akkermansia,* while lean-WD-recipients were enriched in *Coprobacillus, Clostridium, Erysipelotrichaceae, Ruminococcaceae, Lachnospiraceae, Clostridales, Adlercreutzia, Coriobacteriales,* and *Akkermansia*. We also measured significantly enriched taxa based on donor diet since it was an important predictor of tumor outcomes. Compared to LFD-recipients and HFD-recipients, WD-recipients were enriched in *Enterococcus and Bacteroidetes*. Compared to LFD-recipients and WD-recipients, HFD-recipients were enriched in *Sutterella, Dorea, Clostridium* of the *Lachnospiraceae* family and *Clostridia.* Compared to recipients of obesogenic diet FMTs, LFD-recipients were enriched many more taxa including *Allobaculum, Dehalobacterium, Turicibacter, Lactococcus, Lactobacillus, Adlercreutzia,* and *Clostridium* of the *Ruminococcaceae* family.

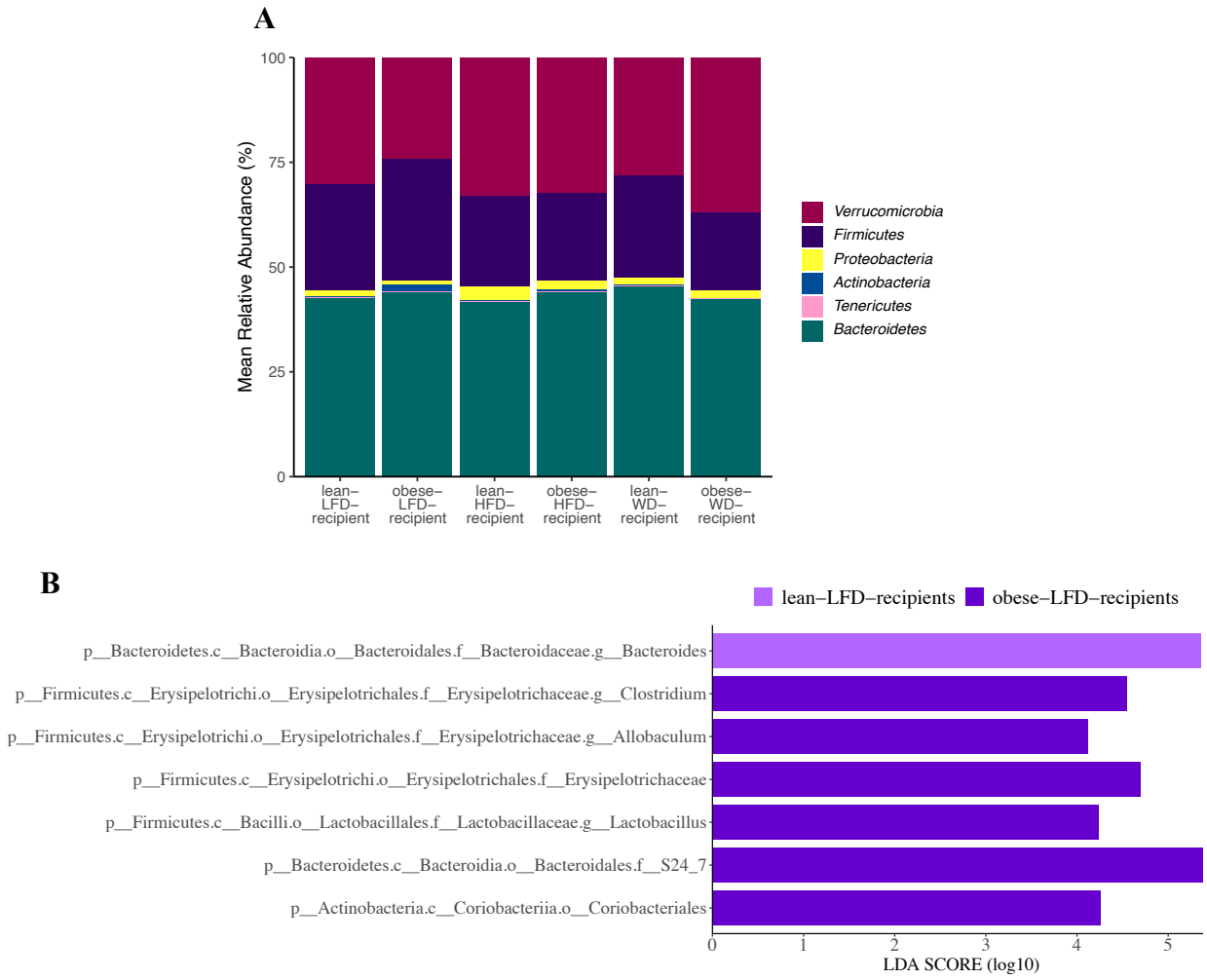
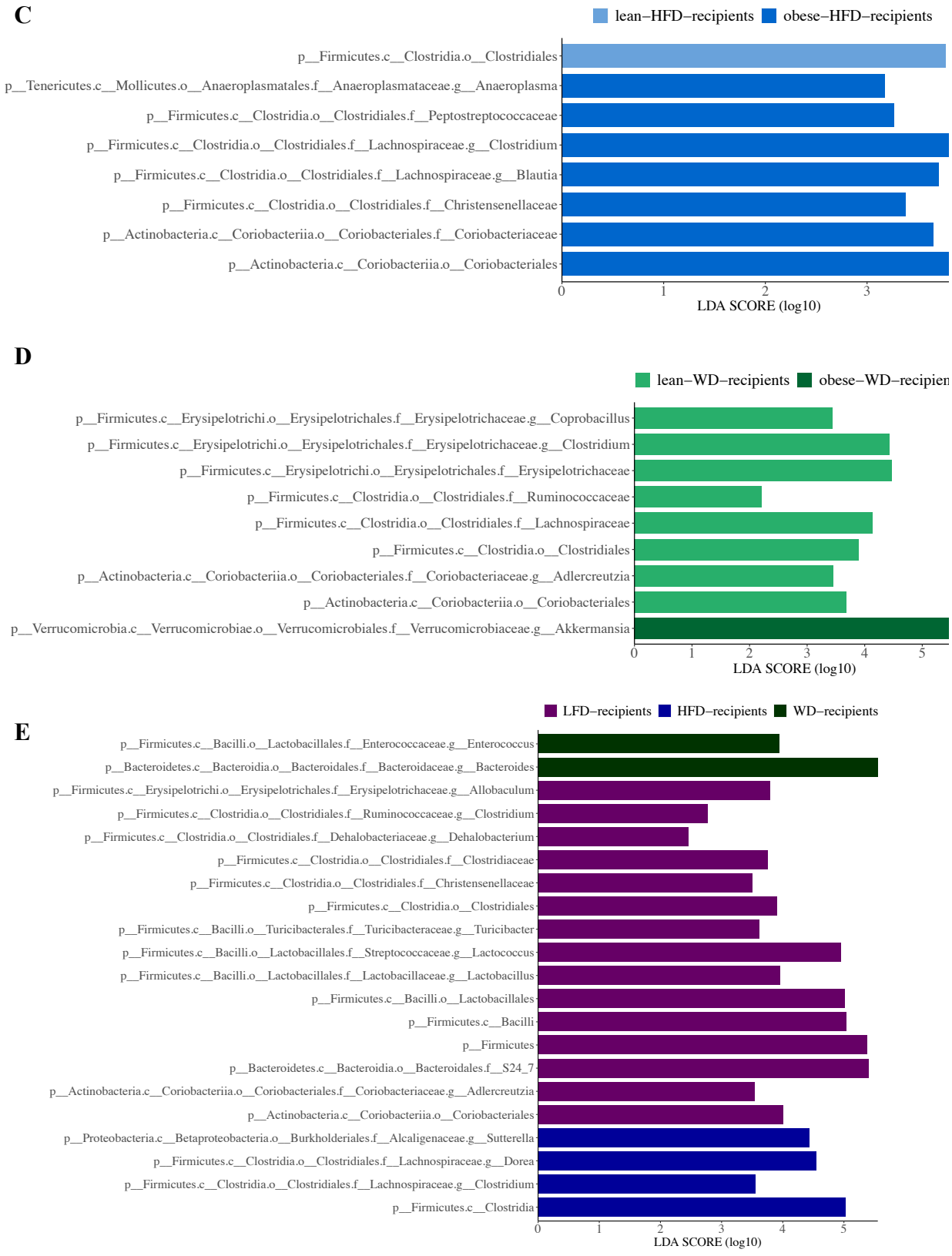


Figure 3.7 Significantly enriched taxa in fecal microbiota prior to tumor development

Fecal samples were collected from male FVB/N recipient mice one week after receiving a fecal microbial transplant (FMT) from lean and obese donor mice that were on a low-fat diet (LFD), high fat diet (HFD) or western diet (WD). (A) percent mean relative abundance of phyla by donor diet. Linear discriminant analysis of effect size (LEfSe) was used to detect differentially abundant bacteria between (B) lean-recipient-LFD and obese-recipient-LFD (C) lean-recipient-HFD and obese-recipient-HFD (D) lean-recipient-WD and obese-recipient-WD and by (E) by donor diet. Length of bar indicates effect size. B-D, n=18-20/group, E, n=36-40/group

Figure 3.7 continued



3.4.6 Alpha diversity in fecal microbiota post-tumor development

The richness and abundance of fecal microbiota collected post-tumor development (fifteen weeks after the last AOM-injection) were assessed. There were interactions between donor diet and donor obesity status in Shannon entropy (Fig 3.8A), Pielou's evenness (Fig 3.8B) and Faith's PD (Fig 3.8D). There appeared to be a greater difference between obese-HFD-recipient and lean-HFD-recipient in Shannon entropy ($p=0.051$), observed features ($p<0.05$) and Pielou's evenness ($p=0.057$) relative to the difference between lean and obese LFD recipients and lean and obese WD recipients. Initially donor obesity status was not associated with significantly less observed features ($p=0.08$). Based on the boxplots, we saw that the difference between the medians of the lean and obese WD and between lean and obese HFD recipients were similar, but different relative to the difference between the medians of the lean and obese-LFD-recipients. This suggested that that the impact of donor obesity-status may be driven by whether the FMT was from a donor on an obesogenic, or not. After recoding the data to test this, we found that recipients given FMTs from obese donors on an obesogenic diet had significantly less observed features relative to recipients given FMTs from lean-recipient counterparts. In all four alpha diversity measurements, there were no differences between the lean-LFD-recipient and obese-LFD-recipients.

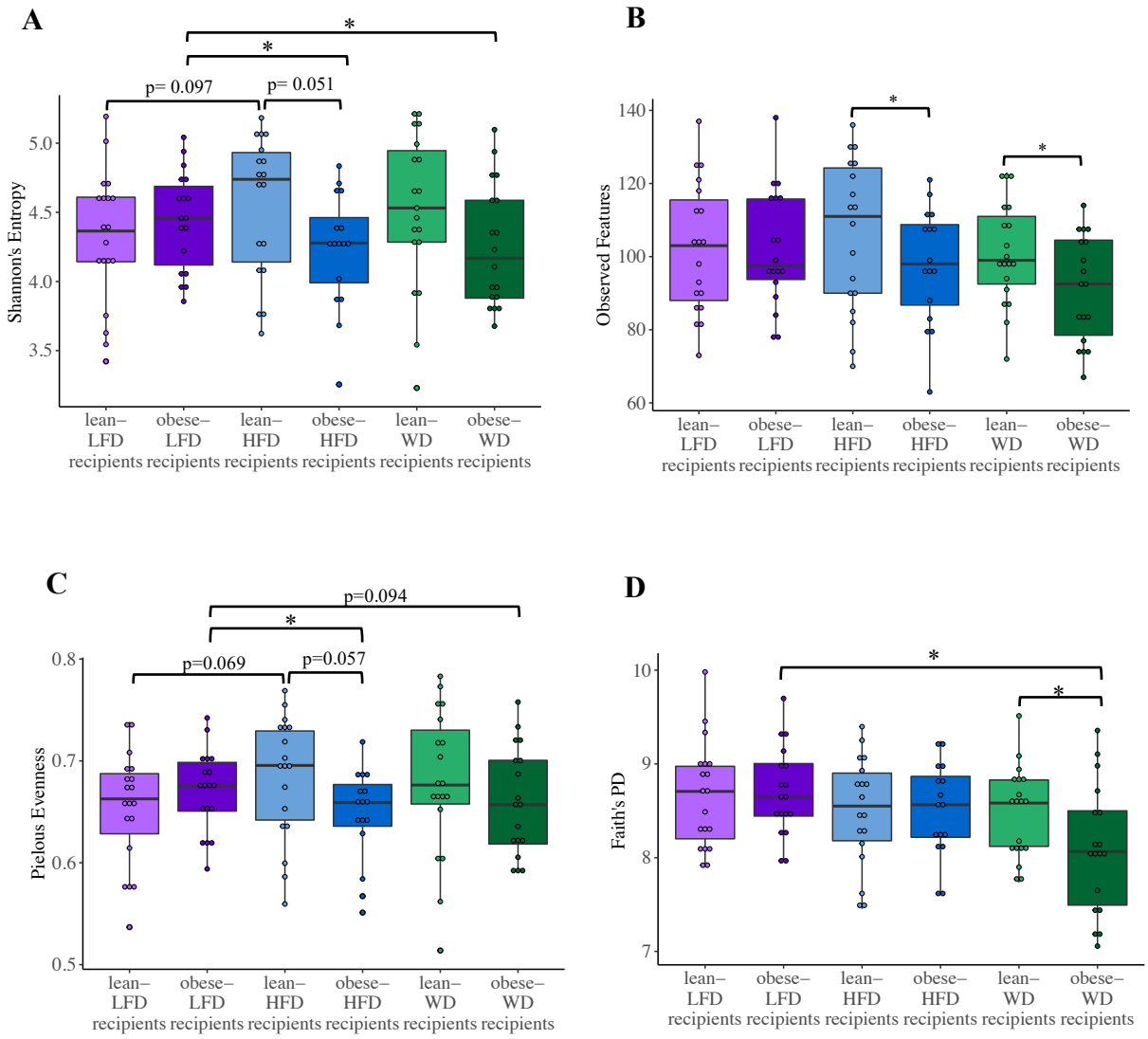


Figure 3.8 Alpha diversity of the fecal microbiota post-tumor development

Colorectal cancer was induced with five weekly injections of azoxymethane (AOM) in male FVB/N mice one week after receiving a fecal microbial transplant (FMT) from lean or obese donor mice that were on a low-fat diet (LFD), high fat diet (HFD) or western diet (WD). Fifteen weeks after the last AOM injection fecal samples were collected and processed for 16S rRNA sequencing (A) Shannon's Entropy (B) observed features (c) Pielou's Evennes (d) Faith's PD. Six groups are colored by diet (LFD=purple, HFD=blue, WD=green), and obesity status (light shade=lean, dark shade=obese), n=18-20/group. Analysis was done using linear regression * $p<0.05$

3.4.7 Beta diversity in fecal microbiota post tumor development

Differences in gut microbial community after tumor development (fifteen weeks after last AOM injection), were calculated using unweighted and weighted UniFrac distances. Unlike beta diversity in pre-tumor fecal microbiota, there was a significant main effect of both obesity ($p<0.02$) (Fig 3.9A) and diet ($p<0.003$) (Fig 3.9B) using both metrics. For ease of identifying differential clustering of microbial communities, unweighted UniFrac distances were used for Fig 3.9A and weighted UniFrac distances were used for Fig. 3.9B. For weighted UniFrac, the LFD-recipients were significantly different ($q< 0.01$) from both the HFD-recipients and WD-recipients, but there was no difference between the HFD-recipients and WD-recipients. (Fig 3.9). For unweighted UniFrac, pair-wise comparisons between the LFD, HFD, and WD recipients were all significant ($p<0.003$), suggesting that the difference in microbial communities between WD and HFD recipients is due to relative abundance (data not shown). Pair-wise comparisons, using weighted and unweighted UniFrac, among the six groups showed that there were significant differences ($q<0.05$) between lean LFD, HFD and WD recipients and their obese LFD, HFD and WD recipient counterparts. In addition, with weighted UniFrac, we detected a significant difference between lean-HFD-recipients and lean-WD-recipients ($q=0.008$), but no difference between obese-HFD-recipients and obese-WD-recipients, suggesting an interaction between donor diet and donor obesity status (data not shown).

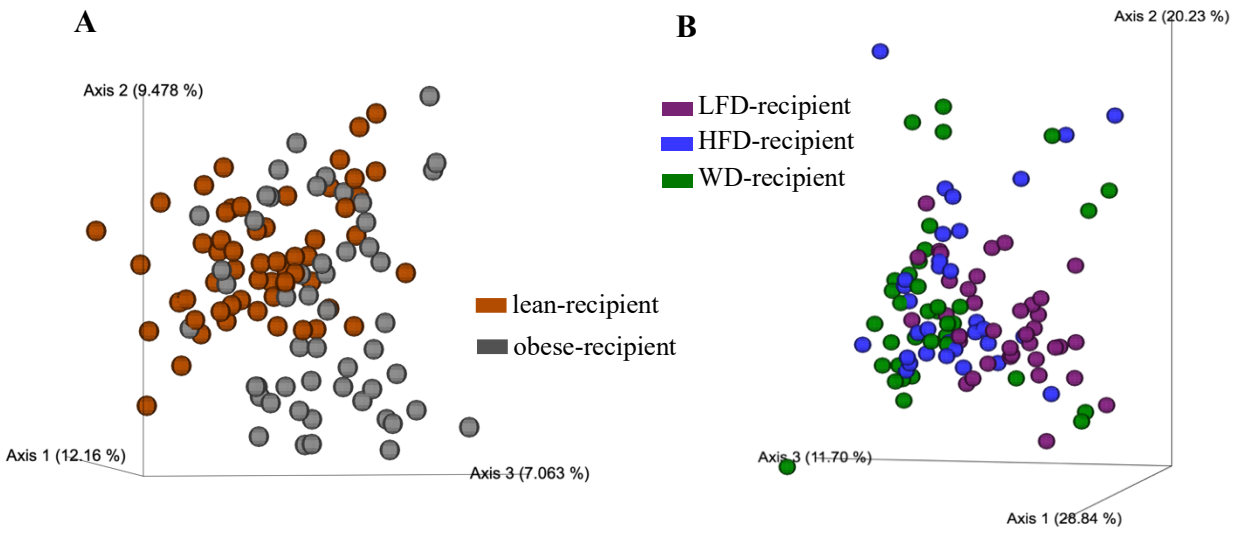


Figure 3.9 Beta diversity of fecal microbiota after tumor development

Colorectal cancer was induced with five weekly injections of azoxymethane (AOM) in male FVB/N mice one week after receiving a fecal microbial transplant (FMT) from lean or obese donor mice that were on a low-fat diet (LFD), high fat diet (HFD) or western diet (WD). Fifteen weeks after the last AOM injection fecal samples were collected and processed for 16S rRNA sequencing. (A) unweighted UniFrac distances were used for principal coordinates analysis (PCoA) to visualize gut microbiota community clustering of recipients by donor obesity status (B) weighted UniFrac distances were used for PCoA to visualize gut microbiota community clustering of recipients by donor diet (n=36-40/group). Significance determined using PERMANOVA followed by pairwise testing, α=0.05.

3.4.8 Significantly enriched taxa in fecal microbiota after tumor development

To determine if donor obesity status influenced taxa enrichment, we first visualized relative abundance by phyla (Fig 3.10A), and then compared lean-LFD-recipients and obese-LFD-recipients (Fig 3.10C), lean-HFD-recipients and obese-HFD-recipients (Fig 3.10D), and lean-WD-recipients and obese-WD-recipients (Fig 3.10D). There were similarities in enrichment based on donor obesity status. For example, both lean-LFD-recipients and lean-WD-recipients were enriched in *RF-39,* and lean-HFD-recipients and lean-WD-recipients were enriched in *Oscillospira*, *Coprococcus,* and *Clostridales.* Both obese-LFD-recipients and obese-HFD-recipients were enriched in *Akkermansia* and *Rikenellaceae*. In addition to similarities between groups, there were also taxa that were enriched in only one of the six groups. For instance, *Turicibacter* was enriched in lean-LFD-recipients; *Mogibacteriaceae* was enriched in obese-LFD-recipients; *S24-7* was enriched in lean-HFD-recipients; *Dorea* was enriched in obese-HFD-

recipients; *Anaeroplasma* was enriched in lean-WD-recipients and *Peptostreptococcaceae* was the only enriched taxa in obese-WD-recipients. There were no taxa enriched in all lean-recipient groups or in all obese-recipient groups. Collectively these results suggest there was an interaction between donor obesity status and donor diet on taxa enrichment.

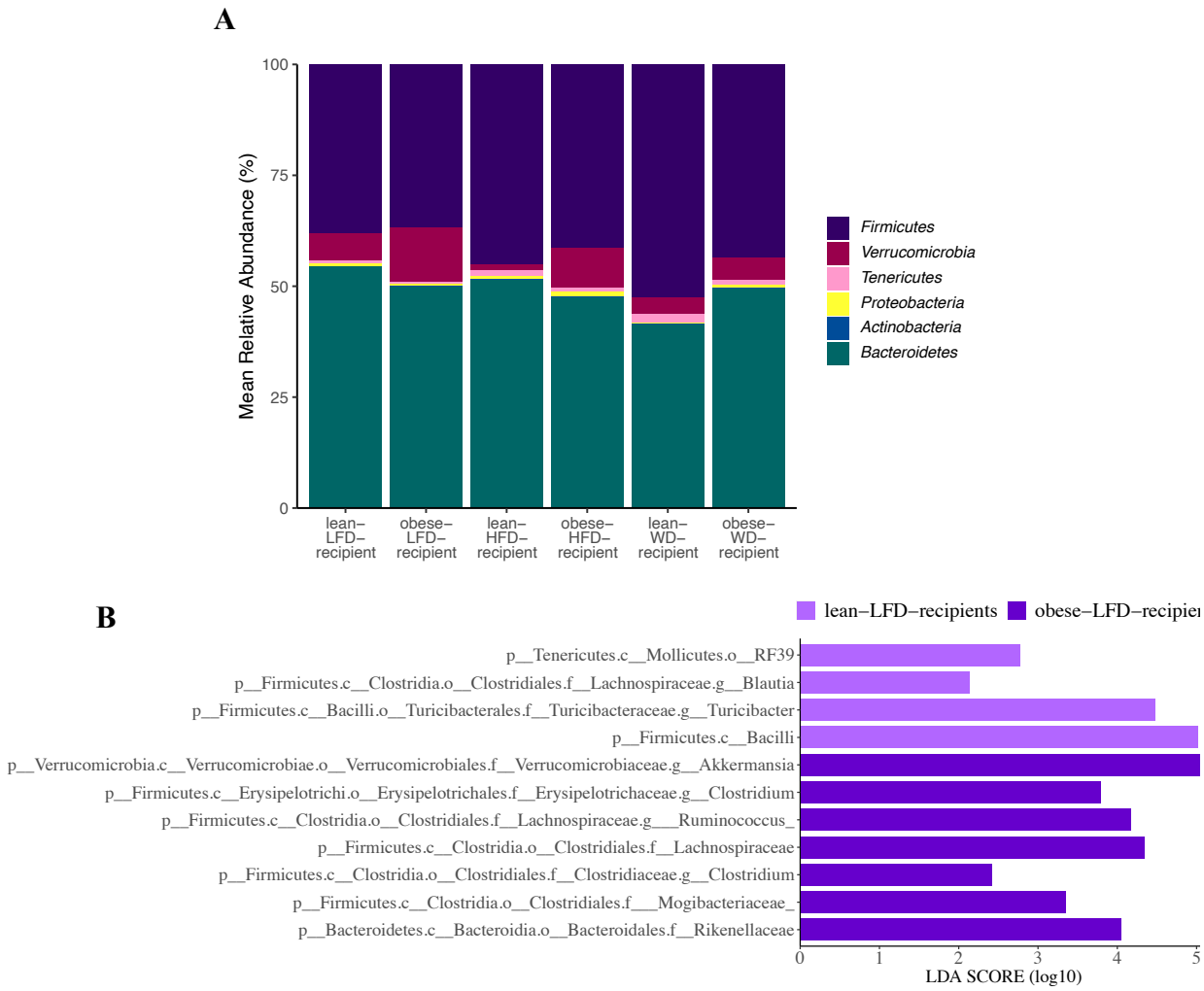
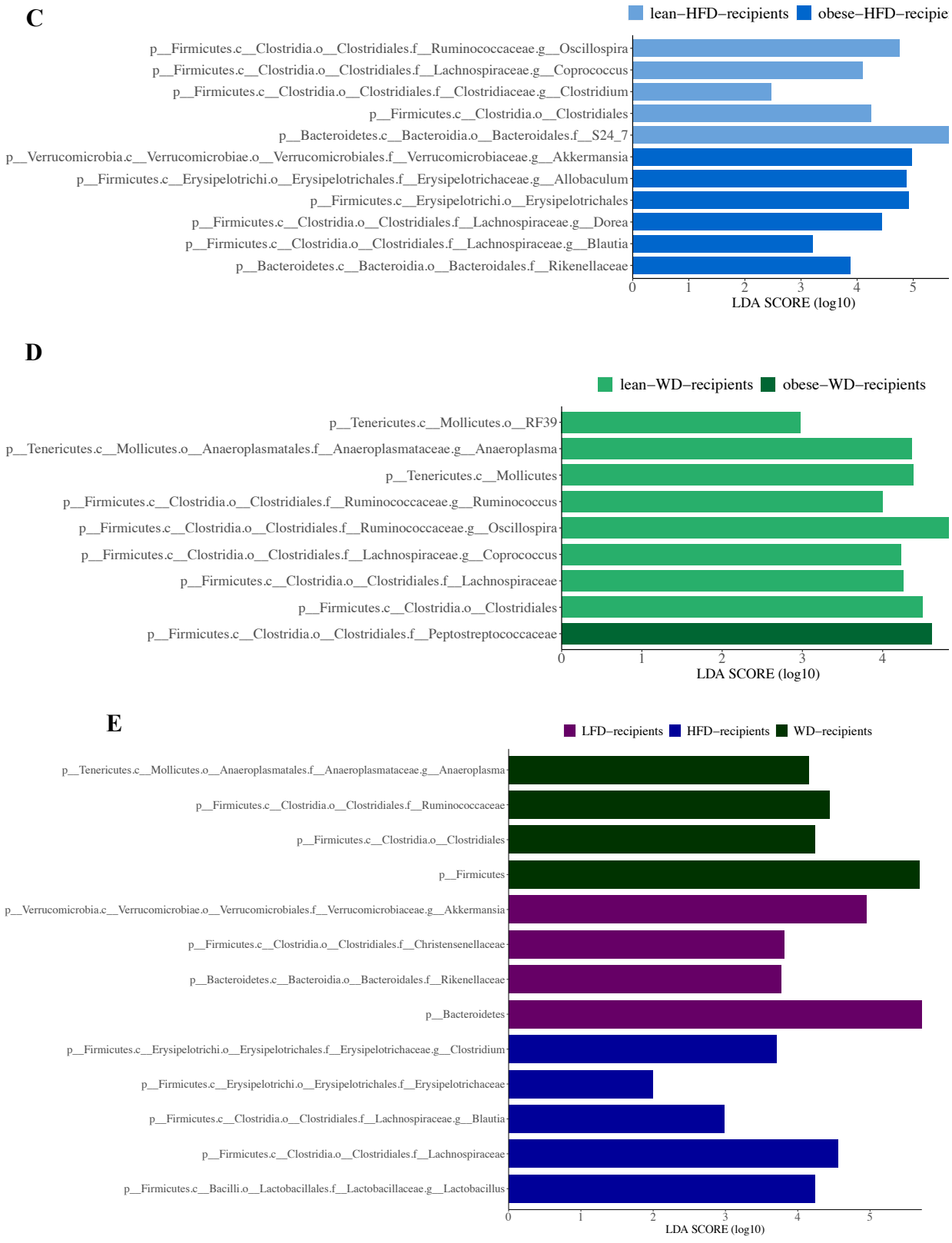


Figure 3.10 Significantly enriched taxa in fecal samples post-tumor development

Colorectal cancer was induced with five weekly injections of azoxymethane (AOM) in male FVB/N mice one week after receiving a fecal microbial transplant (FMT) from lean or obese donor mice that were on a low-fat diet (LFD), high fat diet (HFD) or western diet (WD). Fifteen weeks after the last AOM injection fecal samples were collected and processed for 16S rRNA sequencing. (A) percent mean relative abundance by phylum. Linear discriminant analysis of effect size (LEfSe) was used to detect differentially abundant taxa between (B) lean-recipient-LFD and obese-recipient-LFD (C) lean-recipient-HFD and obese-recipient-HFD (D) lean-recipient-WD and obese-recipient-WD. (E) by donor-diet. Length of bar indicates effect size, n=18-20/group, α= 0.05

Figure 3.10 continued



3.4.9 SIRPα RNA expression was higher in the colon-tumor tissue of WD-recipients

Colon-tumor samples used in histological analysis (Fig 3.4A-E) were also used to measure differentially expressed genes using the NanoString nCounter PanCancer^{TM} IO 360 panel that assesses 730 genes. Considering the relationship between donor diet and tumor outcomes, gene expression was assessed by donor diet. There were no significant differentially regulated genes in the colon-tumor tissue between HFD and LFD recipients or between HFD and WD recipients. In WD-recipients, signal regulatory protein alpha (SIRPα) was significantly increased relative to LFD-recipients (Fig 3.11). This gene codes for an inhibitory receptor expressed by innate immune cells that once activated, inhibits phagocytic action of macrophages and dendritic cells (61).

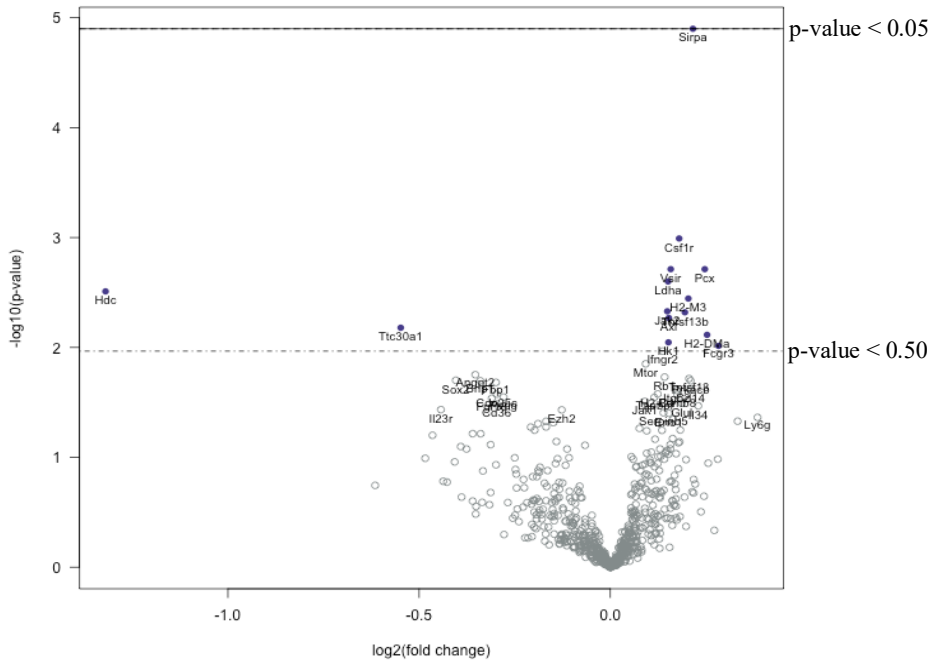


Figure 3.11 Differentially expressed genes in colon-tumor tissue

Colorectal cancer was induced with five weekly injections of azoxymethane (AOM) in male FVB/N mice one week after receiving a fecal microbial transplant (FMT) from donor mice that were on a low-fat diet (LFD), high fat diet (HFD) or western diet (WD). RNA was extracted from colon-tumor tissue for differential gene expression analysis via NanoString nCounter PanCancer^{TM} IO 360 panel. Volcano plot distinguishes significantly enriched genes in WD-recipients compared to LFD-recipients, p-values indicated by dashed line on plot, n=18-20/group. T-tests were performed on log2-transformed count data, and Benjamini-Hochberg correction was used to control for multiple comparisons (α=0.05)

3.4.10 Donor to recipient colonization

To determine the success of donor microbiota colonization in recipient mice, we assessed microbial community composition by plotting weighted UniFrac distances on a PCoA. There was little overlap between the gut microbiota of donors and recipients (pre-tumor) (Fig 3.12). Of the 39 taxa detected, 38 of them were detected in both the donors and recipients (pre-tumor). *Butyricicoccus* was detected in only donor fecal samples.

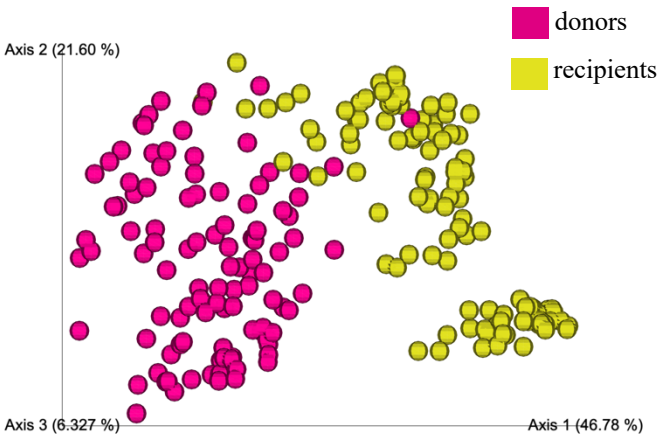


Figure 3.12 Donor-recipient gut microbiota colonization

The gut microbiota from C57BL/6J male mice that developed obesity or remained lean on a low fat diet (LFD), high fat diet (HFD) or western diet (WD) for 15 weeks was transplanted into antibiotic treated FVB/N male mice. Fecal samples were collected one week after colonization and assessed with 16S rRNA gene sequencing. Weighted UniFrac distances were plotted on a PCoA and colored by donor gut microbiota (pink) and recipient microbiota one week after colonization (yellow). Significance determined using PERMANOVA, α=0.05.

3.5 Discussion

In this study, our goal was to isolate the role of obesity-associated gut microbiota on CRC independent from the development of obesity. Using FMTs from lean and obese mice on two obesogenic diets (HFD and WD) and a control diet (LFD), we found that only donor diet (LFD, HFD, WD) and not donor obesity status (lean or obese) was a significant predictor of tumor outcomes. Overall, we found that obesogenic diets influence the gut microbiota to promote CRC.

Like other studies, HFD-recipients were associated with higher tumor incidence and tumor number in the colon and had signs of increased colonic inflammation relative to the control LFD-

recipients (39–41). To explore the role of diet beyond those used in these studies, we also included FMTs from donors on a WD. We measured diet-specific differences in macroscopic and histological outcomes. For example, WD-recipients did not have higher tumor incidence or number compared to LFD-recipients, but they were significantly more likely to have severe, widespread colonic inflammation. WD-recipients also had increased expression of the immosuppressive receptor, signal-regulatory protein alpha (SIRPα). The ligand for SIRPα, CD47, has been found to be overexpressed on human CRC tumors and is correlated with decreased survival (65,66). This demonstrates that the gut microbiota of WD-recipients has factors that can promote and attenuate CRC development such that there is still a higher risk for CRC development relative to LFD-recipients.

Although donor-diet was the main predictor of tumor outcomes, we included assessment of potential effects of obesity-status of the donor on gut microbiota collected before tumor development. We did not detect a significant decrease in alpha diversity of HFD-recipients relative to LFD-recipients like other studies (41,67), but we did measure that WD-recipients had significantly lower alpha diversity compared to HFD-recipients. In all alpha diversity metrics, there were no differences between lean and obese-HFD recipients, while there were significant differences between lean and obese-WD recipients. We found a similar pattern in beta-diversity (weighted UniFrac) in that there were significant differences in microbial communities between lean and obese WD recipients, but not between lean and obese HFD recipients. Based on these results, it may be that HFD-recipients had significantly worse tumor outcomes because the lean and obese donor microbiota was the same, amplifying its effect in tumorigenesis. On the other hand, the microbiota of lean and obese WD-recipients was significantly different, increasing the likelihood for the presence of microbiota that may attenuate CRC development.

To further understand dietary effects on donor microbiota and CRC outcomes, we analyzed significantly enriched taxa by donor diet. HFD-recipients were enriched in *Dorea* and *Sutterella*, which have been detected in studies of HFD-induced obesity (68,69). These genera have also been measured in the gut microbiota of CRC patients, are associated with later stages of the disease, and can adhere to CRC cells (70–74). WD-recipients were enriched in *Bacteroides* and *Enterococcus. Bacteroides* has been found to be positively associated with Westernized countries that consume diets high in animal protein and saturated fat (75–78). Although these dietary habits are linked to increased CRC risk, potential effects of these bacteria vary widely based on species

and strain. For example, *Bacteroides fragilis,* is a bile-resistant commensal organism commonly found in the gut. However, some strains produce an enterotoxin which has been shown to promote CRC in mice and is enriched in CRC patients (26,27,30,79). Similarly, *Enterococcus faecalis* is a common commensal of the gut that has been shown to protect against tumorigenesis in rats, but has also been implicated in promoting intestinal permeability, reactive oxygen species, DNA damage, and is enriched in both lean and obese CRC patients (80–86).

After tumor development, gut microbiota changed. Unlike alpha diversity measured in fecal microbiota before tumor development, we measured significant differences between lean and obese HFD recipients and between lean and obese WD recipients. Unlike beta diversity measured in fecal microbiota before tumor development, we measured a significant main effect of both donor diet and donor obesity status. Based on weighted UniFrac distances, LFD-recipients had significantly different microbial communities compared to HFD-recipients and WD-recipients, while there were no statistical differences between microbial communities of HFD and WD recipients. After tumor development, HFD-recipients were enriched in *Blautia*, a genus commonly measured in obesity (87,88) and associated with processed food and intestinal inflammation (89). Contrarily, they were also significantly enriched in *Lactobacillus* which has been shown to have anti-cancer activities (90,91) and is negatively associated with CRC tumors (92). Members of the *Erysipelotrichaceae* family are enriched in CRC patients (93) and in animal models of CRC (92) was also increased in HFD-recipients. The only genera enriched in WD-recipients was *Anaeroplasma.* This bacterium has been associated with promoting anti-inflammatory mechanisms in the colon (94), but was also enriched in HFD-fed mice that developed CRC (95). Although we did not detect bacteria highly linked to CRC such as *Fusobacterium nucleatum*, pks^{+} *Escherichia coli*, or enterotoxigenic *Bacteroidetes fragilis* (ETBF) (29), our results demonstrate that microbes present and enriched in this model may influence CRC development.

In this study, we have shown that while obesity can influence gut microbiota composition, it is primarily obesogenic diets that influence CRC through changing the gut microbiota. Differences in tumorigenesis present in HFD-recipients and WD-recipients demonstrate that diet composition is critical in any gut microbiota mediated effects. However, there were limitations. Most taxa present in the donors were present in the recipients one week after colonization, but overall microbial community composition between donors and colonized recipients was significantly different. Thus, it is difficult to determine if diet and obesity related microbes from

donors were adequately transplanted into recipients. This may be because microbes in the FMT that were sensitive to environmental changes did not survive the freeze thaw cycle of the FMT aliquot or the digestive process inherent in FMT administration through oral gavage. Using a 1:1 donor-to-recipient FMT strategy allowed us to identify any potential microbial community outliers, but it also likely contributed to higher microbial community variation within each FMT recipient group, thereby decreasing our ability to detect significance in some CRC outcomes at $\alpha = 0.05$. In future studies, we may be able to decrease this variation by pooling FMTs for each of the treatment groups. While the usefulness of a priori power calculations for providing a rough idea of the sample size required to detect differences at $\alpha = 0.05$ is widely accepted, the practice of attempting to explain the observed data using post-hoc power analyses is controversial (96). In particular, if the combination of high post-hoc power and a non-rejected null hypothesis is taken as evidence for the apparent truth of the null, one must also accept the flawed conclusion that smaller but non-significant p-values provide more evidence for the null than larger p-values do (see section 2.2 of Hoenig & Heisey). If the results of the present studies are to be used to inform the sample size to be used in follow-up experiments, it is important to note that the effect sizes estimated should not be assumed to be the true values of the underlying population, and that the models used, like all models used in applied sciences, are likely to be misspecified.

Although modeling the diversity of human dietary patterns is difficult, our study has highlighted the importance of diet composition in gut microbiota and CRC risk. It is only to our detriment if most results from animal models were based on slight variations of a diet high in fat. As such, further research concerning the role of dietary effects on gut microbiota in CRC risk and development would be instrumental in designing inventions and strategies that would reduce the risk for this disease.

3.6 Acknowledgements

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3.7 References

1. Abar L, Vieira AR, Aune D, Sobiecki JG, Vingeliene S, Polemiti E, et al. Height and body fatness and colorectal cancer risk: an update of the WCRF–AICR systematic review of published prospective studies. Eur J Nutr. 2018 Aug;57(5):1701–20.

2. Siegel RL, Miller KD, Goding Sauer A, Fedewa SA, Butterly LF, Anderson JC, et al. Colorectal cancer statistics, 2020. CA Cancer J Clin. 2020 May;70(3):145–64.

3. Virostko J, Capasso A, Yankeelov TE, Goodgame B. Recent trends in the age at diagnosis of colorectal cancer in the US National Cancer Data Base, 2004-2015. Cancer. 2019 Nov;125(21):3828–35.

4. Araghi M, Soerjomataram I, Bardot A, Ferlay J, Cabasag CJ, Morrison DS, et al. Changes in colorectal cancer incidence in seven high-income countries: a population-based study. Lancet Gastroenterol Hepatol. 2019 Jul;4(7):511–8.

5. Ward ZJ, Long MW, Resch SC, Giles CM, Cradock AL, Gortmaker SL. Simulation of Growth Trajectories of Childhood Obesity into Adulthood. N Engl J Med. 2017 Nov 30;377(22):2145–53.

6. Ward ZJ, Bleich SN, Cradock AL, Barrett JL, Giles CM, Flax C, et al. Projected U.S. State-Level Prevalence of Adult Obesity and Severe Obesity. N Engl J Med. 2019 Dec 19;381(25):2440–50.

7. Larsson SC, Wolk A. Obesity and colon and rectal cancer risk: a meta-analysis of prospective studies. Am J Clin Nutr. 2007 Sep 1;86(3):556–65.

8. Moghaddam AA, Woodward M, Huxley R. Obesity and Risk of Colorectal Cancer: A Meta-analysis of 31 Studies with 70,000 Events. Cancer Epidemiol Biomarkers Prev. 2007 Dec 1;16(12):2533–47.

9. O'Neill AM, Burrington CM, Gillaspie EA, Lynch DT, Horsman MJ, Greene MW. High-fat Western diet–induced obesity contributes to increased tumor growth in mouse models of human colon cancer. Nutr Res. 2016 Dec;36(12):1325–34.

10. Olivo-Marston SE, Hursting SD, Perkins SN, Schetter A, Khan M, Croce C, et al. Effects of calorie restriction and diet-induced obesity on murine colon carcinogenesis, growth and inflammatory factors, and microRNA expression. PloS One. 2014;9(4):e94765.

11. Rondini EA, Harvey AE, Steibel JP, Hursting SD, Fenton JI. Energy balance modulates colon tumor growth: Interactive roles of insulin and estrogen. Mol Carcinog. 2011 May;50(5):370–82.

12. Day SD, Enos RT, McClellan JL, Steiner JL, Velázquez KT, Murphy EA. Linking inflammation to tumorigenesis in a mouse model of high-fat-diet-enhanced colon cancer. Cytokine. 2013 Oct;64(1):454–62.

13. Pendyala S, Neff LM, Suárez-Fariñas M, Holt PR. Diet-induced weight loss reduces colorectal inflammation: implications for colorectal carcinogenesis. Am J Clin Nutr. 2011 Feb;93(2):234–42.

14. Fried SK, Bunkin DA, Greenberg AS. Omental and Subcutaneous Adipose Tissues of Obese Subjects Release Interleukin-6: Depot Difference and Regulation by Glucocorticoid [1]. J Clin Endocrinol Metab. 1998 Mar;83(3):847–50.

15. Murano I, Barbatelli G, Parisani V, Latini C, Muzzonigro G, Castellucci M, et al. Dead adipocytes, detected as crown-like structures, are prevalent in visceral fat depots of genetically obese mice. J Lipid Res. 2008 Jul;49(7):1562–8.

16. Fontana L, Eagon JC, Trujillo ME, Scherer PE, Klein S. Visceral fat adipokine secretion is associated with systemic inflammation in obese humans. Diabetes. 2007 Apr;56(4):1010–3.

17. Lumeng CN, Deyoung SM, Bodzin JL, Saltiel AR. Increased inflammatory properties of adipose tissue macrophages recruited during diet-induced obesity. Diabetes. 2007 Jan;56(1):16–23.

18. Garrett WS. Immune recognition of microbial metabolites. Nat Rev Immunol. 2020 Feb;20(2):91–2.

19. Li L, Li X, Zhong W, Yang M, Xu M, Sun Y, et al. Gut microbiota from colorectal cancer patients enhances the progression of intestinal adenoma in Apcmin/+ mice. EBioMedicine. 2019 Oct;48:301–15.

20. Wong SH, Zhao L, Zhang X, Nakatsu G, Han J, Xu W, et al. Gavage of Fecal Samples From Patients With Colorectal Cancer Promotes Intestinal Carcinogenesis in Germ-Free and Conventional Mice. Gastroenterology. 2017 Dec;153(6):1621-1633.e6.

21. Bäckhed F, Ding H, Wang T, Hooper LV, Koh GY, Nagy A, et al. The gut microbiota as an environmental factor that regulates fat storage. Proc Natl Acad Sci U S A. 2004 Nov 2;101(44):15718–23.

22. Bäckhed F, Manchester JK, Semenkovich CF, Gordon JI. Mechanisms underlying the resistance to diet-induced obesity in germ-free mice. Proc Natl Acad Sci U S A. 2007 Jan 16;104(3):979–84.

23. Cani PD, Bibiloni R, Knauf C, Waget A, Neyrinck AM, Delzenne NM, et al. Changes in Gut Microbiota Control Metabolic Endotoxemia-Induced Inflammation in High-Fat Diet–Induced Obesity and Diabetes in Mice. Diabetes. 2008 Jun 1;57(6):1470–81.

24. Ridaura VK, Faith JJ, Rey FE, Cheng J, Duncan AE, Kau AL, et al. Gut Microbiota from Twins Discordant for Obesity Modulate Metabolism in Mice. Science. 2013 Sep 6;341(6150):1241214.

25. Kostic AD, Chun E, Robertson L, Glickman JN, Gallini CA, Michaud M, et al. Fusobacterium nucleatum Potentiates Intestinal Tumorigenesis and Modulates the Tumor-Immune Microenvironment. Cell Host Microbe. 2013 Aug;14(2):207–15.

26. Rubinstein MR, Baik JE, Lagana SM, Han RP, Raab WJ, Sahoo D, et al. *Fusobacterium nucleatum* promotes colorectal cancer by inducing Wnt/β-catenin modulator Annexin A1. EMBO Rep [Internet]. 2019 Apr [cited 2022 Jan 12];20(4). Available from: https://onlinelibrary.wiley.com/doi/10.15252/embr.201847638

27. Arthur JC, Perez-Chanona E, Mühlbauer M, Tomkovich S, Uronis JM, Fan TJ, et al. Intestinal Inflammation Targets Cancer-Inducing Activity of the Microbiota. Science. 2012 Oct 5;338(6103):120–3.

28. Haghi F, Goli E, Mirzaei B, Zeighami H. The association between fecal enterotoxigenic B. fragilis with colorectal cancer. BMC Cancer. 2019 Dec;19(1):879.

29. Garrett WS. The gut microbiota and colon cancer. Science. 2019 Jun 21;364(6446):1133–5.

30. Bullman S, Pedamallu CS, Sicinska E, Clancy TE, Zhang X, Cai D, et al. Analysis of *Fusobacterium* persistence and antibiotic response in colorectal cancer. Science. 2017 Dec 15;358(6369):1443–8.

31. Zackular JP, Baxter NT, Iverson KD, Sadler WD, Petrosino JF, Chen GY, et al. The gut microbiome modulates colon tumorigenesis. mBio. 2013 Nov 5;4(6):e00692-00613.

32. Zackular JP, Baxter NT, Chen GY, Schloss PD. Manipulation of the Gut Microbiota Reveals Role in Colon Tumorigenesis. mSphere. 2016 Feb;1(1):e00001-15.

33. Burrello C, Garavaglia F, Cribiù FM, Ercoli G, Lopez G, Troisi J, et al. Therapeutic faecal microbiota transplantation controls intestinal inflammation through IL10 secretion by immune cells. Nat Commun. 2018 Dec;9(1):5184.

34. Kong C, Gao R, Yan X, Huang L, Qin H. Probiotics improve gut microbiota dysbiosis in obese mice fed a high-fat or high-sucrose diet. Nutrition. 2019 Apr;60:175–84.

35. Roller M, Femia AP, Caderni G, Rechkemmer G, Watzl B. Intestinal immunity of rats with colon cancer is modulated by oligofructose-enriched inulin combined with Lactobacillus rhamnosus and Bifidobacterium lactis. Br J Nutr. 2004 Dec;92(6):931–8.

36. De Palma G, Lynch MDJ, Lu J, Dang VT, Deng Y, Jury J, et al. Transplantation of fecal microbiota from patients with irritable bowel syndrome alters gut function and behavior in recipient mice. Sci Transl Med. 2017 Mar 1;9(379):eaaf6397.

37. Alard J, Lehrter V, Rhimi M, Mangin I, Peucelle V, Abraham AL, et al. Beneficial metabolic effects of selected probiotics on diet-induced obesity and insulin resistance in mice are associated with improvement of dysbiotic gut microbiota: Probiotics, obesity and gut microbiota. Environ Microbiol. 2016 May;18(5):1484–97.

38. Burrello C, Giuffrè MR, Macandog AD, Diaz-Basabe A, Cribiù FM, Lopez G, et al. Fecal Microbiota Transplantation Controls Murine Chronic Intestinal Inflammation by Modulating Immune Cell Functions and Gut Microbiota Composition. Cells. 2019 May 28;8(6):517.

39. Schulz MD, Atay Ç, Heringer J, Romrig FK, Schwitalla S, Aydin B, et al. High-fat-diet-mediated dysbiosis promotes intestinal carcinogenesis independently of obesity. Nature. 2014 Oct;514(7523):508–12.

40. Liu T, Guo Z, Song X, Liu L, Dong W, Wang S, et al. High-fat diet-induced dysbiosis mediates MCP-1/CCR2 axis-dependent M2 macrophage polarization and promotes intestinal adenoma-adenocarcinoma sequence. J Cell Mol Med. 2020 Feb;24(4):2648–62.

41. Yang J, Wei H, Zhou Y, Szeto CH, Li C, Lin Y, et al. High-Fat Diet Promotes Colorectal Tumorigenesis Through Modulating Gut Microbiota and Metabolites. Gastroenterology. 2022 Jan;162(1):135-149.e2.

42. Kastl AJ, Terry NA, Wu GD, Albenberg LG. The Structure and Function of the Human Small Intestinal Microbiota: Current Understanding and Future Directions. Cell Mol Gastroenterol Hepatol. 2020;9(1):33–45.

43. Anders JL, Moustafa MAM, Mohamed WMA, Hayakawa T, Nakao R, Koizumi I. Comparing the gut microbiome along the gastrointestinal tract of three sympatric species of wild rodents. Sci Rep. 2021 Dec;11(1):19929.

44. Rowland I, Gibson G, Heinken A, Scott K, Swann J, Thiele I, et al. Gut microbiota functions: metabolism of nutrients and other food components. Eur J Nutr. 2018 Feb;57(1):1–24.

45. Lomba A, Milagro FI, García-Díaz DF, Marti A, Campión J, Martínez JA. Obesity induced by a pair-fed high fat sucrose diet: methylation and expression pattern of genes related to energy homeostasis. Lipids Health Dis. 2010 Dec;9(1):60.

46. Turnbaugh PJ, Ridaura VK, Faith JJ, Rey FE, Knight R, Gordon JI. The Effect of Diet on the Human Gut Microbiome: A Metagenomic Analysis in Humanized Gnotobiotic Mice. Sci Transl Med [Internet]. 2009 Nov 11 [cited 2022 Jan 1];1(6). Available from: https://www.science.org/doi/10.1126/scitranslmed.3000322

47. Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Appl Environ Microbiol. 2013 Sep;79(17):5112–20.

48. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol. 2019 Aug;37(8):852–7.

49. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods. 2016 Jul;13(7):581–3.

50. Callahan BJ, McMurdie PJ, Holmes SP. Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. ISME J. 2017 Dec;11(12):2639–43.

51. McDonald D, Price MN, Goodrich J, Nawrocki EP, DeSantis TZ, Probst A, et al. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. ISME J. 2012 Mar;6(3):610–8.

52. Bokulich NA, Kaehler BD, Rideout JR, Dillon M, Bolyen E, Knight R, et al. Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. Microbiome. 2018 Dec;6(1):90.

53. Shannon CE. A Mathematical Theory of Communication. Bell Syst Tech J. 1948 Jul;27(3):379–423.

54. Faith DP. Conservation evaluation and phylogenetic diversity. Biol Conserv. 1992;61(1):1–10.

55. Pielou EC. The measurement of diversity in different types of biological collections. J Theor Biol. 1966 Dec;13:131–44.

56. Lozupone C, Knight R. UniFrac: a New Phylogenetic Method for Comparing Microbial Communities. Appl Environ Microbiol. 2005 Dec;71(12):8228–35.

57. Lozupone CA, Hamady M, Kelley ST, Knight R. Quantitative and Qualitative β Diversity Measures Lead to Different Insights into Factors That Structure Microbial Communities. Appl Environ Microbiol. 2007 Mar;73(5):1576–85.

58. Benjamini Y, Hochberg Y. Controlling The False Discovery Rate - A Practical And Powerful Approach To Multiple Testing. Journal of the Royal Statistical Society Series B: Methodological. 1995 Nov;57:289–300.

59. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, et al. Metagenomic biomarker discovery and explanation. Genome Biol. 2011;12(6):R60.

60. R Core Team. R: A Language and Environment for Statistical Computing [Internet]. Vienna, Austria: R Foundation for Statistical Computing; 2021. Available from: https://www.R-project.org/

61. Firth D. Bias reduction of maximum likelihood estimates. Biometrika. 1993;80(1):27–38.

62. Heinze G, Schemper M. A solution to the problem of separation in logistic regression. Stat Med. 2002 Aug 30;21(16):2409–19.

63. Morgan-Wall T, Khoury G. Optimal Design Generation and Power Evaluation in *R* : The **skpr** Package. J Stat Softw [Internet]. 2021 [cited 2022 Apr 24];99(1). Available from: http://www.jstatsoft.org/v99/i01/

64. Nuzzo R. Scientific method: Statistical errors. Nature. 2014 Feb 13;506(7487):150–2.

65. Takahashi S. Molecular functions of SIRPα and its role in cancer (Review). Biomed Rep [Internet]. 2018 May 23 [cited 2022 Feb 17]; Available from: http://www.spandidos-publications.com/10.3892/br.2018.1102

66. Willingham SB, Volkmer JP, Gentles AJ, Sahoo D, Dalerba P, Mitra SS, et al. The CD47-signal regulatory protein alpha (SIRPa) interaction is a therapeutic target for human solid tumors. Proc Natl Acad Sci. 2012 Apr 24;109(17):6662–7.

67. Xiao L, Sonne SB, Feng Q, Chen N, Xia Z, Li X, et al. High-fat feeding rather than obesity drives taxonomical and functional changes in the gut microbiota in mice. Microbiome. 2017 Dec;5(1):43.

68. Companys J, Gosalbes MJ, Pla-Pagà L, Calderón-Pérez L, Llauradó E, Pedret A, et al. Gut Microbiota Profile and Its Association with Clinical Variables and Dietary Intake in Overweight/Obese and Lean Subjects: A Cross-Sectional Study. Nutrients. 2021 Jun 13;13(6):2032.

69. Ottosson F, Brunkwall L, Ericson U, Nilsson PM, Almgren P, Fernandez C, et al. Connection Between BMI-Related Plasma Metabolite Profile and Gut Microbiota. J Clin Endocrinol Metab. 2018 Apr 1;103(4):1491–501.

70. Ho CL, Tan HQ, Chua KJ, Kang A, Lim KH, Ling KL, et al. Engineered commensal microbes for diet-mediated colorectal-cancer chemoprevention. Nat Biomed Eng. 2018 Jan;2(1):27–37.

71. Hiippala K, Kainulainen V, Kalliomäki M, Arkkila P, Satokari R. Mucosal Prevalence and Interactions with the Epithelium Indicate Commensalism of Sutterella spp. Front Microbiol [Internet]. 2016 Oct 26 [cited 2022 Feb 16];7. Available from: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01706/full

72. Mori G, Rampelli S, Orena BS, Rengucci C, De Maio G, Barbieri G, et al. Shifts of Faecal Microbiota During Sporadic Colorectal Carcinogenesis. Sci Rep. 2018 Dec;8(1):10329.

73. Zhang M, Lv Y, Hou S, Liu Y, Wang Y, Wan X. Differential Mucosal Microbiome Profiles across Stages of Human Colorectal Cancer. Life. 2021 Aug 13;11(8):831.

74. Hibberd AA, Lyra A, Ouwehand AC, Rolny P, Lindegren H, Cedgård L, et al. Intestinal microbiota is altered in patients with colon cancer and modified by probiotic intervention. BMJ Open Gastroenterol. 2017 Jul;4(1):e000145.

75. Wu GD, Chen J, Hoffmann C, Bittinger K, Chen YY, Keilbaugh SA, et al. Linking Long-Term Dietary Patterns with Gut Microbial Enterotypes. Science. 2011 Oct 7;334(6052):105–8.

76. Klimenko N, Tyakht A, Popenko A, Vasiliev A, Altukhov I, Ischenko D, et al. Microbiome Responses to an Uncontrolled Short-Term Diet Intervention in the Frame of the Citizen Science Project. Nutrients. 2018 May 8;10(5):576.

77. Gorvitovskaia A, Holmes SP, Huse SM. Interpreting Prevotella and Bacteroides as biomarkers of diet and lifestyle. Microbiome. 2016 Dec;4(1):15.

78. Tomova A, Bukovsky I, Rembert E, Yonas W, Alwarith J, Barnard ND, et al. The Effects of Vegetarian and Vegan Diets on Gut Microbiota. Front Nutr. 2019 Apr 17;6:47.

79. Wu S, Rhee KJ, Albesiano E, Rabizadeh S, Wu X, Yen HR, et al. A human colonic commensal promotes colon tumorigenesis via activation of T helper type 17 T cell responses. Nat Med. 2009 Sep;15(9):1016–22.

80. Sivieri K, Spinardi-Barbisan ALT, Barbisan LF, Bedani R, Pauly ND, Carlos IZ, et al. Probiotic Enterococcus faecium CRL 183 inhibit chemically induced colon cancer in male Wistar rats. Eur Food Res Technol. 2008 Dec;228(2):231–7.

81. Huycke MM, Abrams V, Moore DR. Enterococcus faecalis produces extracellular superoxide and hydrogen peroxide that damages colonic epithelial cell DNA. Carcinogenesis. 2002 Mar;23(3):529–36.

82. Jacobson A, Lam L, Rajendram M, Tamburini F, Honeycutt J, Pham T, et al. A Gut Commensal-Produced Metabolite Mediates Colonization Resistance to Salmonella Infection. Cell Host Microbe. 2018 Aug;24(2):296-307.e7.

83. Steck N, Hoffmann M, Sava IG, Kim SC, Hahne H, Tonkonogy SL, et al. Enterococcus faecalis Metalloprotease Compromises Epithelial Barrier and Contributes to Intestinal Inflammation. Gastroenterology. 2011 Sep;141(3):959–71.

84. Are A, Aronsson L, Wang S, Greicius G, Lee YK, Gustafsson JA, et al. Enterococcus faecalis from newborn babies regulate endogenous PPAR activity and IL-10 levels in colonic epithelial cells. Proc Natl Acad Sci. 2008 Feb 12;105(6):1943–8.

85. Wang X, Allen TD, May RJ, Lightfoot S, Houchen CW, Huycke MM. *Enterococcus faecalis* Induces Aneuploidy and Tetraploidy in Colonic Epithelial Cells through a Bystander Effect. Cancer Res. 2008 Dec 1;68(23):9909–17.

86. Sánchez-Alcoholado L, Ordóñez R, Otero A, Plaza-Andrade I, Laborda-Illanes A, Medina JA, et al. Gut Microbiota-Mediated Inflammation and Gut Permeability in Patients with Obesity and Colorectal Cancer. Int J Mol Sci. 2020 Sep 16;21(18):6782.

87. Kasai C, Sugimoto K, Moritani I, Tanaka J, Oya Y, Inoue H, et al. Comparison of the gut microbiota composition between obese and non-obese individuals in a Japanese population, as analyzed by terminal restriction fragment length polymorphism and next-generation sequencing. BMC Gastroenterol. 2015 Dec;15(1):100.

88. Ozato N, Saito S, Yamaguchi T, Katashima M, Tokuda I, Sawada K, et al. Blautia genus associated with visceral fat accumulation in adults 20–76 years of age. Npj Biofilms Microbiomes. 2019 Dec;5(1):28.

89. Bolte LA, Vich Vila A, Imhann F, Collij V, Gacesa R, Peters V, et al. Long-term dietary patterns are associated with pro-inflammatory and anti-inflammatory features of the gut microbiome. Gut. 2021 Jul;70(7):1287–98.

90. Sun M, Liu W, Song Y, Tuo Y, Mu G, Ma F. The Effects of Lactobacillus plantarum-12 Crude Exopolysaccharides on the Cell Proliferation and Apoptosis of Human Colon Cancer (HT-29) Cells. Probiotics Antimicrob Proteins. 2021 Apr;13(2):413–21.

91. Zhang T, Pan D, Yang Y, Jiang X, Zhang J, Zeng X, et al. Effect of *Lactobacillus acidophilus* CICC 6074 S-Layer Protein on Colon Cancer HT-29 Cell Proliferation and Apoptosis. J Agric Food Chem. 2020 Mar 4;68(9):2639–47.

92. Zhu Q, Jin Z, Wu W, Gao R, Guo B, Gao Z, et al. Analysis of the Intestinal Lumen Microbiota in an Animal Model of Colorectal Cancer. Hold GL, editor. PLoS ONE. 2014 Mar 6;9(3):e90849.

93. Chen W, Liu F, Ling Z, Tong X, Xiang C. Human Intestinal Lumen and Mucosa-Associated Microbiota in Patients with Colorectal Cancer. Moschetta A, editor. PLoS ONE. 2012 Jun 28;7(6):e39743.

94. Beller A, Kruglov A, Durek P, von Goetze V, Hoffmann U, Maier R, et al. P104 Anaeroplasma, a potential anti-inflammatory probiotic for the treatment of chronic intestinal inflammation. In: Abstracts [Internet]. BMJ Publishing Group Ltd and European League Against Rheumatism; 2019 [cited 2022 Feb 17]. p. A45.2-A46. Available from: https://ard.bmj.com/lookup/doi/10.1136/annrheumdis-2018-EWRR2019.92

95. Zeng H, Ishaq SL, Liu Z, Bukowski MR. Colonic aberrant crypt formation accompanies an increase of opportunistic pathogenic bacteria in C57BL/6 mice fed a high-fat diet. J Nutr Biochem. 2018 Apr;54:18–27.

96. Hoenig JM, Heisey DM. The Abuse of Power: The Pervasive Fallacy of Power Calculations for Data Analysis. Am Stat. 2001 Feb;55(1):19–24.

CHAPTER 4. CONCLUSIONS AND FUTURE DIRECTIONS

4.1 Summary

In the United States, colorectal cancer (CRC) incidence in younger populations has doubled since the 1990s (1). Researchers have predicted that by 2030, 11% of colon cancers and 23% of rectal cancers will be diagnosed in adults younger than 50 years old, highlighting the need to understand factors that may be contributing to this trend (1–3). One modifiable lifestyle factor that has been consistently associated with CRC risk is obesity (4,5). Obesity is a multifaceted condition characterized by metabolic and physiologic conditions such as insulin resistance, increased circulating hormone concentration and chronic inflammation that can promote cancer initiation and progression (6–9). These pro-tumorigenic signaling factors stem in part from excess adipose tissue populated by hypertrophic adipocytes and inflammatory macrophages (10–14). Considering the location of CRC, another factor that has been shown to be an important mediator of CRC development is the gut microbiota (15–18).

The colon houses trillions of commensal microbes that interact with the colonic mucosa and luminal contents to maintain homeostasis (19–21). Obesity is associated with shifts in the gut microbiota concurrent with worsening colonic health which increases the risk for CRC. This is modeled in mice where increased inflammation, intestinal permeability, and colonic tumorigenesis are enhanced in diet induced obesity (22–25). However, any effects due to the gut microbiota are confounded by other obesity-related changes, so the independent effects of the gut microbiota in obesity associated colonic tumorigenesis is unclear. Within the gut microbiota-obesity-CRC relationship, diet is an important covariate because it drives the development of obesity, shapes gut microbiota composition, and has itself been associated with mediating CRC risk (26–29). Given this omnipresent role, further research into the effect of diet is necessary. To determine if obesity and/or diet influences gut microbiota composition to mediate CRC development, we performed the following studies.

In the first study (Chapter 2), we generated lean and obese mice on a LFD, HFD or WD to determine if diet and/or obesity influenced gut microbiota composition and intestinal permeability. Diet and obesity status significantly impacted alpha diversity, a measurement of richness and/or abundance of a microbial community. In all metrics used, WD and HFD mice had higher alpha

diversity compared to the LFD mice, and WD mice had higher alpha diversity compared to the HFD mice. We also found that compared to their lean counterparts, obesity on the LFD and WD was associated with a greater decrease in richness compared obese mice on the HFD. In beta-diversity, which assesses differences between microbial communities, we found that remaining lean on the HFD and WD resulted in two distinct clusters compared to obese mice on the HFD and WD whose microbial communities displayed more variation and overlap. This pattern was also identified in results from the *in vivo* test of intestinal permeability using FITC-dextran. Lean-HFD mice and lean-WD mice displayed better barrier function compared to the lean-LFD mice, while there were no differences among obese groups. These results suggest that the gut microbiota affected by HFD and WD in the absence of obesity may promote a stronger intestinal barrier.

In the second study (Chapter 3), we aimed to determine if obesity and/or diet mediated changes to the gut microbiota independently influence the development of CRC. Fecal and cecal content harvested from mice from Chapter 2 was used as donor microbiota to colonize antibiotic-treated recipient mice that were then given the chemical carcinogen, azoxymethane, to induce CRC. We showed that diet status of the donor mice was a significant predictor of CRC outcomes. HFD-recipients had increased tumor incidence and tumor number relative to LFD-recipients, and WD-recipients had significantly worse colonic inflammation compared to LFD-recipients. Prior to tumor development, WD and HFD-recipients were significantly enriched in different genera. Some species within these genera have been shown to promote and/or prevent CRC development such as *Enterococcus, Bacteroides* and *Sutterella* (30–35). Overall, this study demonstrates the critical role diet composition plays in gut microbial effects on CRC.

Collectively, we show that obesity status and diet significantly influence gut microbial composition and intestinal permeability. However, only dietary effects on gut microbiota composition influence CRC development. Considering diet is a modifiable lifestyle factor relevant to all humans, further research into dietary effects on the gut microbiota in impacting CRC development is critical to identify effective methods in prevention and treatment.

4.2 Future directions

4.2.1 Gut microbiota and intestinal barrier function

In the first study (Chapter 2) we found that the gut microbiota of lean-HFD and lean-WD mice may be responsible for promoting significantly better gut barrier function relative to the gut microbiota of lean-LFD. This discovery suggests that bacterial taxa enriched in both lean-HFD and lean-WD groups can influence intestinal permeability. For instance, *Coprobacillus* was significantly enriched in lean mice on the WD and HFD. Damage to the intestinal epithelium was associated with decreased *Coprobacillus* (36), and increased *Coprobacillus* was associated with butyrate supplementation that promoted restoration of the intestinal epithelium (37). Altogether, published research and our findings support the potential role of *Coprobacillus* in improving the gut barrier. Therefore, we hypothesize that *Coprobacillus* promotes intestinal barrier integrity. To date, there is only one identified, culturable species of *Coprobacillus, catenaformis* (38). Since our results also demonstrated that the obese-HFD and obese-WD had higher gut permeability relative to their lean counterparts, we can inoculate obese-HFD and obese-WD mice with *Coprobacillus catenaformis* and then measure intestinal permeability with *in vivo* FITC-dextran, serum lipopolysaccharide protein (LPS), and expression of mucus and tight junction proteins. We expect that mice on the HFD and WD that were inoculated with *Coprobacillus catenaformis* to have better barrier integrity and have gut microbial communities enriched in this bacterium compared to obese mice on the WD and HFD that did not receive *Coprobacillus catenaformis.*

To further understand the role of *Coprobacillus catenaformis* in intestinal barrier function, we could also measure its effects in healing the colonic epithelium. Conventional mice on a semi-purified, low-fat diet would be given drinking water supplemented with dextran sodium sulfate (DSS), a chemical that ulcerates and damages the colon. After one week of this treatment, we would then inoculate mice with *Coprobacillus catenaformis* and measure intestinal permeability 7 days and 14 days later to measure rate of healing (39). We would also euthanize mice at these time points to perform histological assessment and IHC staining to measure cellular signs of epithelial recovery. In addition, we would collect fecal samples at baseline, after DSS and at both 7- and 14-days post DSS to determine the relative abundance of *Coprobacillus catenaformis* throughout this process. We expect that mice that were treated with DSS and *Coprobacillus catenaformis* will display a faster rate of intestinal healing compared to mice not supplemented

with this bacterium. These results may aid in elucidating specific bacteria that could be used to help treat intestinal inflammation to reduce CRC risk.

4.2.2 Sugar effects in microbiota mediated CRC

In the second study (Chapter 3), we found that HFD-recipients and WD-recipients did not develop CRC in the same manner. It was interesting to find that WD-recipients did not have worse macroscopic tumor outcomes (tumor incidence and number) relative to LFD-recipients because a different study that transplanted gut microbiota from donors on a diet with a similar percentage and source of fat into recipient mice did observe significantly worse colon tumor number. This suggests that the higher percentage of sucrose in the WD in our study may have attenuated HFD effects on microbiota mediated promotion of CRC development. To further explore sucrose effects on the gut microbiota in attenuating CRC, we would use the same experimental set-up in Chapter 2 and Chapter 3, except donors would be on one of four diets: (1) high fat (HFD) (2) western diet (high fat+high sucrose) (3) high sucrose (HS) or (4) control low fat diet (matched for sucrose in the HFD). Similar to the results in Chapter 2 and 3, we would expect that compared to control LFD-recipients, HFD-recipients will have worse tumor outcomes relative to the WD-recipients. HS-recipients will have comparable tumor outcomes to controls but have significantly different microbiota. Measuring taxa enriched in the gut microbiota of both HS-recipients and WD-recipients will help narrow down potential bacteria that may be involved in slowing the progression of CRC.

The role of sucrose in mediating CRC development through the gut microbiota can be further explored by addressing different effects on microbial community composition due to the route of administration- through diet or drinking water. Past research has shown that when delivered through diet, high concentrations of sucrose and fructose do not induce obesity, but when provided through drinking water, do promote the development of obesity and related metabolic conditions (40–42). Observing different obesity-related effects mediated by sugar delivered through diet or drinking water prompted the question of whether sucrose administered through diet had a different effect on gut microbiota composition compared to administration through drinking water and whether these gut microbial changes influence the development of CRC. In one study, controlled administration of high fructose corn syrup through oral gavage to prevent the development of obesity and obesity associated metabolic syndrome still resulted in increased CRC

development (43). Similarly, in AOM-induced CRC, oral gavage of sucrose and fructose was shown to increase colonic cell proliferation and formation of ACF compared to mice that received an oral gavage of glucose and water (44). However, these studies did not measure the gut microbiota and thus could not determine if there were differences between diet and liquid administration of sugar on gut microbiota mediated effects of CRC. To determine if there are differential effects to gut microbial communities when sucrose is administered through drinking water or diet and whether these microbial changes influence CRC development, we would use the same study design used in Chapter 2 and Chapter 3. Donor mice would be on one of 4 treatments (1) *ad libitum* access to a high sucrose diet (2) *ad libitum* access to sucrose in drinking water and a (3) controlled access to sucrose in drinking water to prevent weight gain and (4) control diet with no sucrose. Although we did not measure obesity-status of the donor to be a significant predictor of CRC outcomes, we do not know if this is the case when nutrients are not administered through diet. We would expect that the method of sucrose administration does influence gut microbiota composition, but that gut microbiota associated effects on CRC development would not be dependent on method of sucrose administration. Thus, recipients of the microbiota from both sucrose groups would be associated with increased colonic inflammation and other measurements of poor intestinal health compared to recipients of the gut microbiota from mice on the LFD. These results would lend insight into whether the two major sources of sugar, diet and beverage, impact the gut microbiota in comparable ways to promote conditions in the colon that influence risk for CRC.

4.3 Conclusions

In this work we have shown the importance of diet and obesity on gut microbiota composition and intestinal permeability. In particular, we demonstrated that obesogenic diet effects on the gut microbiota significantly influence CRC development and that obesity-mediated effects on the gut microbiota had little to no effect in promoting this disease. Moreover, the extent of microbial effects on CRC was dependent on diet composition. In sum, this work improves our understanding of the role of diet and gut microbiota on CRC development. Further research in this field will aid in designing effective strategies that use diet to help reverse the rising incidence of CRC in young adults.

4.4 References

1. Bailey CE, Hu C-Y, You YN, Bednarski BK, Rodriguez-Bigas MA, Skibber JM, et al. Increasing disparities in the age-related incidences of colon and rectal cancers in the United States, 1975-2010. JAMA Surg. 2015 Jan;150(1):17–22.

2. Siegel RL, Miller KD, Goding Sauer A, Fedewa SA, Butterly LF, Anderson JC, et al. Colorectal cancer statistics, 2020. CA Cancer J Clin. 2020 May;70(3):145–64.

3. Stoffel EM, Murphy CC. Epidemiology and Mechanisms of the Increasing Incidence of Colon and Rectal Cancers in Young Adults. Gastroenterology. 2020 Jan;158(2):341–53.

4. Larsson SC, Wolk A. Obesity and colon and rectal cancer risk: a meta-analysis of prospective studies. Am J Clin Nutr. 2007 Sep 1;86(3):556–65.

5. Abar L, Vieira AR, Aune D, Sobiecki JG, Vingeliene S, Polemiti E, et al. Height and body fatness and colorectal cancer risk: an update of the WCRF–AICR systematic review of published prospective studies. Eur J Nutr. 2018 Aug;57(5):1701–20.

6. Endo H, Hosono K, Uchiyama T, Sakai E, Sugiyama M, Takahashi H, et al. Leptin acts as a growth factor for colorectal tumours at stages subsequent to tumour initiation in murine colon carcinogenesis. Gut. 2011 Oct 1;60(10):1363–71.

7. Koda M, Sulkowska M, Kanczuga-Koda L, Surmacz E, Sulkowski S. Overexpression of the obesity hormone leptin in human colorectal cancer. J Clin Pathol. 2007 Aug;60(8):902–6.

8. Kern L, Mittenbühler MJ, Vesting AJ, Ostermann AL, Wunderlich CM, Wunderlich FT. Obesity-Induced TNFα and IL-6 Signaling: The Missing Link between Obesity and Inflammation-Driven Liver and Colorectal Cancers. Cancers. 2018 Dec 27;11(1):E24.

9. Makki K, Froguel P, Wolowczuk I. Adipose tissue in obesity-related inflammation and insulin resistance: cells, cytokines, and chemokines. ISRN Inflamm. 2013 Dec 22;2013:139239.

10. Fried SK, Bunkin DA, Greenberg AS. Omental and Subcutaneous Adipose Tissues of Obese Subjects Release Interleukin-6: Depot Difference and Regulation by Glucocorticoid [1]. J Clin Endocrinol Metab. 1998 Mar;83(3):847–50.

11. Samad F, Yamamoto K, Pandey M, Loskutoff DJ. Elevated expression of transforming growth factor-beta in adipose tissue from obese mice. Mol Med Camb Mass. 1997 Jan;3(1):37–48.

12. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW. Obesity is associated with macrophage accumulation in adipose tissue. J Clin Invest. 2003 Dec 15;112(12):1796–808.

13. Cancello R, Henegar C, Viguerie N, Taleb S, Poitou C, Rouault C, et al. Reduction of Macrophage Infiltration and Chemoattractant Gene Expression Changes in White Adipose Tissue of Morbidly Obese Subjects After Surgery-Induced Weight Loss. Diabetes. 2005 Aug 1;54(8):2277–86.

14. Curat CA, Wegner V, Sengenès C, Miranville A, Tonus C, Busse R, et al. Macrophages in human visceral adipose tissue: increased accumulation in obesity and a source of resistin and visfatin. Diabetologia. 2006 Apr;49(4):744–7.

15. Hanahan D. Hallmarks of Cancer: New Dimensions. Cancer Discov. 2022 Jan;12(1):31–46.

16. Garrett WS. The gut microbiota and colon cancer. Science. 2019 Jun 21;364(6446):1133–5.

17. Zackular JP, Baxter NT, Chen GY, Schloss PD. Manipulation of the Gut Microbiota Reveals Role in Colon Tumorigenesis. mSphere. 2016 Feb;1(1):e00001-15.

18. Li L, Li X, Zhong W, Yang M, Xu M, Sun Y, et al. Gut microbiota from colorectal cancer patients enhances the progression of intestinal adenoma in Apcmin/+ mice. EBioMedicine. 2019 Oct;48:301–15.

19. Garrett WS. Immune recognition of microbial metabolites. Nat Rev Immunol. 2020 Feb;20(2):91–2.

20. Sonnenburg JL, Bäckhed F. Diet–microbiota interactions as moderators of human metabolism. Nature. 2016 Jul 7;535(7610):56–64.

21. Hill MJ. Intestinal flora and endogenous vitamin synthesis: Eur J Cancer Prev. 1997 Mar;6:S43–5.

22. Zhu Q-C. Effect of a high-fat diet in development of colonic adenoma in an animal model. World J Gastroenterol. 2014;20(25):8119.

23. Olivo-Marston SE, Hursting SD, Perkins SN, Schetter A, Khan M, Croce C, et al. Effects of calorie restriction and diet-induced obesity on murine colon carcinogenesis, growth and inflammatory factors, and microRNA expression. PloS One. 2014;9(4):e94765.

24. O'Neill AM, Burrington CM, Gillaspie EA, Lynch DT, Horsman MJ, Greene MW. High-fat Western diet–induced obesity contributes to increased tumor growth in mouse models of human colon cancer. Nutr Res. 2016 Dec;36(12):1325–34.

25. Cani PD, Bibiloni R, Knauf C, Waget A, Neyrinck AM, Delzenne NM, et al. Changes in Gut Microbiota Control Metabolic Endotoxemia-Induced Inflammation in High-Fat Diet–Induced Obesity and Diabetes in Mice. Diabetes. 2008 Jun 1;57(6):1470–81.

26. De Filippo C, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, Massart S, et al. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proc Natl Acad Sci. 2010 Aug 17;107(33):14691–6.

27. Walker AW, Ince J, Duncan SH, Webster LM, Holtrop G, Ze X, et al. Dominant and diet-responsive groups of bacteria within the human colonic microbiota. ISME J. 2011 Feb;5(2):220–30.

28. David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, et al. Diet rapidly and reproducibly alters the human gut microbiome. Nature. 2014 Jan;505(7484):559–63.

29. O'Keefe SJD. Diet, microorganisms and their metabolites, and colon cancer. Nat Rev Gastroenterol Hepatol. 2016 Dec;13(12):691–706.

30. Hibberd AA, Lyra A, Ouwehand AC, Rolny P, Lindegren H, Cedgård L, et al. Intestinal microbiota is altered in patients with colon cancer and modified by probiotic intervention. BMJ Open Gastroenterol. 2017 Jul;4(1):e000145.

31. Ho CL, Tan HQ, Chua KJ, Kang A, Lim KH, Ling KL, et al. Engineered commensal microbes for diet-mediated colorectal-cancer chemoprevention. Nat Biomed Eng. 2018 Jan;2(1):27–37.

32. Hiippala K, Kainulainen V, Kalliomäki M, Arkkila P, Satokari R. Mucosal Prevalence and Interactions with the Epithelium Indicate Commensalism of Sutterella spp. Front Microbiol [Internet]. 2016 Oct 26 [cited 2022 Feb 16];7. Available from: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01706/full

33. Mori G, Rampelli S, Orena BS, Rengucci C, De Maio G, Barbieri G, et al. Shifts of Faecal Microbiota During Sporadic Colorectal Carcinogenesis. Sci Rep. 2018 Dec;8(1):10329.

34. Zhang M, Lv Y, Hou S, Liu Y, Wang Y, Wan X. Differential Mucosal Microbiome Profiles across Stages of Human Colorectal Cancer. Life. 2021 Aug 13;11(8):831.

35. Wu S, Rhee K-J, Albesiano E, Rabizadeh S, Wu X, Yen H-R, et al. A human colonic commensal promotes colon tumorigenesis via activation of T helper type 17 T cell responses. Nat Med. 2009 Sep;15(9):1016–22.

36. Yu L, Yu Y, Yin R, Duan H, Qu D, Tian F, et al. Dose-dependent effects of lead induced gut injuries: An in vitro and in vivo study. Chemosphere. 2021 Mar;266:129130.

37. Ye J, Lv L, Wu W, Li Y, Shi D, Fang D, et al. Butyrate Protects Mice Against Methionine–Choline-Deficient Diet-Induced Non-alcoholic Steatohepatitis by Improving Gut Barrier Function, Attenuating Inflammation and Reducing Endotoxin Levels. Front Microbiol. 2018 Aug 21;9:1967.

38. Kageyama A, Benno Y. *Coprobacillus catenaformis* Gen. Nov., Sp. Nov., a New Genus and Species Isolated from Human Feces. Microbiol Immunol. 2000 Jan;44(1):23–8.

39. Vidal-Lletjós S, Andriamihaja M, Blais A, Grauso M, Lepage P, Davila A-M, et al. Mucosal healing progression after acute colitis in mice. World J Gastroenterol. 2019 Jul 21;25(27):3572–89.

40. Glendinning JI, Breinager L, Kyrillou E, Lacuna K, Rocha R, Sclafani A. Differential effects of sucrose and fructose on dietary obesity in four mouse strains. Physiol Behav. 2010 Oct;101(3):331–43.

41. Kanarek RB, Orthen-Gambill N. Differential Effects of Sucrose, Fructose and Glucose on Carbohydrate-Induced Obesity in Rats. J Nutr. 1982 Aug 1;112(8):1546–54.

42. Cao D, Lu H, Lewis TL, Li L. Intake of Sucrose-sweetened Water Induces Insulin Resistance and Exacerbates Memory Deficits and Amyloidosis in a Transgenic Mouse Model of Alzheimer Disease. J Biol Chem. 2007 Dec;282(50):36275–82.

43. Goncalves MD, Lu C, Tutnauer J, Hartman TE, Hwang S-K, Murphy CJ, et al. High-fructose corn syrup enhances intestinal tumor growth in mice. Science. 2019 Mar 22;363(6433):1345–9.

44. Stamp D, Zhang X-M, Medline A, Bruce WR, Archer MC. Sucrose enhancement of the early steps of colon carcinogenesis in mice. Carcinogenesis. 1993;14(4):777–9.