DISSERTATION

SUPPLEMENTING POWDERED HIGH-FIBER FOODS TO ALTER GUT MICROBIAL METABOLISM FOR COLORECTAL CANCER PREVENTION

Submitted by

Amy Marie Sheflin

Department of Food Science and Human Nutrition

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Summer 2016

Doctoral Committee:

Advisor: Tiffany Weir

Corey Broeckling
Chris Melby
Michael Pagliassotti

Copyright by Amy Marie Sheflin 2016

All Rights Reserved

ABSTRACT

SUPPLEMENTING POWDERED HIGH-FIBER FOODS TO ALTER GUT MICROBIAL METABOLISM FOR COLORECTAL CANCER PREVENTION

Trillions of microorganisms populate the human digestive tract and enhance immunity, improve digestion and inhibit pathogens when in healthy balance. However, a disruption of gut community balance is associated with a number of diseases including colorectal cancer (CRC). The purpose of this research was to investigate supplementation with two high-fiber foods, heat stabilized rice bran (SRB) and cooked navy bean powder (NBP), for potential to favorably alter the intestinal environment for CRC prevention. Study 1 aimed to assess differences in stool microbiota and associated metabolites in healthy individuals versus those diagnosed with CRC. Next, two pilot dietary interventions were undertaken (Study 2 and 3), one in healthy individuals and another in CRC survivors. Both pilot studies provided diets supplemented with SRB or NBP or macronutrient matched control foods and changes in stool microbiota and associated metabolites were assessed at baseline and after 2 and 4 weeks. The collective body of this research supports the hypothesis that gut microbiota and their metabolites differ with respect to CRC and that intervention with SRB or NBP can alter this profile.

Despite similar increases in dietary fiber intake, SRB induced more significant change to gut microbiota and metabolites possibly due to differing chemical composition of plant fibers. Relative to NBP, SRB is rich in arabinoxylans, which structurally mimic the protective mucins lining the intestinal surface. Changes in both microbiota and metabolites observed with SRB supplementation are consistent with enrichment of glycan-foraging bacteria able to switch

between dietary glycans (arabinoxylans in this case) and host glycans for energy. Acetate and propionate, potentially beneficial byproducts of microbial fermentation of glycans and other fiber components, increased at 2 weeks in CRC survivors. However, some of the observed changes did not persist at 4 weeks and further research is necessary to determine if more lasting changes are possible with these dietary interventions, as well as whether these altered microbial/metabolite profiles are associated with reduced risk of CRC incidence and recurrence.

ACKNOWLEDGEMENTS

In the middle of our campus at Colorado State University is a larger-than-life metal sculpture complete with a stone bench that is engraved with this quote from Sir Isaac Newton, "*If I have seen further than others, it is by standing upon the shoulders of giants*." I have been fortunate to have many giants to lead the way for me during my graduate education. First, I would like to thank my advisor, Dr. Tiffany Weir. I truly had a Most Excellent Advisor in Dr. Weir, because she has provided the perfect balance of sage-like advice and freedom to explore my own ideas and hunches. I will dearly miss our many exciting brainstorming sessions, troubleshooting huddles, and a-ha! moments that have been part of my being on her research team. I am also grateful for my 'Dream Team' Graduate Committee: Drs. Corey Broeckling, Chris Melby, and Mike Pagliassotti. Each of them saw greatness in me at times that I could not see it in myself and through their vision I kept moving forward in spite of my own doubts. Each of these individuals also provided resources and guidance indispensible to my finishing this PhD program, I will be forever grateful to each of them for all they have given to me.

If there is one person that I am impossibly more grateful for than even these amazing advisors it is my one-of-a-kind, beyond-compassionate husband, John. John has tolerated more than any husband should have to tolerate in the name of science including long-winded conversations about the world-saving superpowers of bacteria and tear-filled grievances of science gone wrong. He is my emotional rock, my best friend, and my soul mate. John, thank you for making it possible for me to chase my unicorns. You earned this PhD every bit as much as I did and if I could give it to you, in honor, I would.

Johnny and Elwood, I know having a mother in graduate school for many of your formative years has not been easy. There were events that were missed, details overlooked, and long days waiting at the lab for Mom to finish her work. You both truly know about balancing needs in a family even when yours aren't at the top and you learned it at a young age. I am so proud of both of you and if you learn anything from my graduate school experience I hope it is to believe in your own potential and to stretch yourself to contribute to the world in the way that you love the most.

To my parents, I know you haven't always understood what drives my wild wanderings and I'm sure it was hard for you to see me under so much stress as I've challenged myself over many years. You are the largest giants of all, because you loved me and encouraged me and valued me before anyone else did. It's because of you that I am not afraid of anything and think I can do all the exciting things in this wide world that I might want to try. It's a rare gift you gave me and I thank you for it.

I am incredibly blessed with a wide support net of incredibly inspiring and talented family and friends that are too many to list by name (but I think you know who you are). Some live near and others farther away, some are older than me and some much, much younger, and some I see often, some only rarely. Yet, each of you has given me something unique to fuel me along this journey that it's impossible to put into words. To all of you dear kindred spirits - thank you.

I also have some more formal thanks to make. This research was supported with funding from National Institutes of Health (NIHR21CA161472), the Colorado Agriculture Experiment Station, and International Life Sciences Institute. I would also like to thank CSU Proteomics and Metabolomics Facility for their support for this work and time spent educating me in

understanding metabolomics data. I also appreciate the CSU nutrition science group, Daniel K. Manter (USDA-ARS), and fellow students in the Weir lab for camaraderie and helpful comments on data interpretation/analysis.

TABLE OF CONTENTS

CHAPTER 1: LITERATURE REVIEW

Importance of whole grains and legumes in food security and human health

Rice (*Oryza sativa*) is the staple food of more than half the world's population including three of the world's most populous nations — China, India, and Indonesia, which together have nearly 3 billion people. Rice is important for world food security in that it is an affordable and readily available crop to the poorest of people. To heighten awareness of the role of rice in alleviating poverty and malnutrition, the 57th session of the United Nations (UN) General Assembly designated 2004 as 'The Year of Rice'. In addition, the UN reaffirmed the need to focus world attention on the role rice can play in providing food security. Here in the USA, the average person consumes about 25 pounds of rice per year, of which about 4 pounds is used for brewing beer. However, Americans now eat about twice as much rice as they did 10 years ago, which highlights the increasing domestic importance of this food crop [1].

In contrast, consumption of pulses (grain legumes) has been steadily decreasing over the last several decades except in the Near East/North Africa region [2]. However, pulses offer enormous potential for world food security as an economically accessible and highly nutritious protein source. In addition, pulses promote sustainable agricultural practices by promoting biodiversity and enriching nitrogen content of the soil. This year the UN General Assembly declared 2016 'The International Year of Pulses' in order to "*heighten public awareness of the nutritional benefits of pulses as part of sustainable food production aimed towards food security and nutrition"*. Pulses contain amino acids that are lacking in cereal grains, which serve to raise protein quality when these two foods are consumed together. In addition, pulses are rich in iron and zinc making them particularly beneficial for those at risk of iron-deficiency anemia. Pulses

and legumes were recently recommended to improve the quality of the US diet in part for their mineral content, including iron [3]. Iron has been identified as a 'shortfall' nutrient in the US because it is under-consumed by many adolescent girls and premenopausal women [4].

Diets that consistently contain whole grains and legumes are associated with lower risk for developing multiple diseases including cardiovascular disease, cancer, obesity, and diabetes [5-8]. Risk for cancers of the gastrointestinal tract is particularly sensitive to diet since these tissues are in frequent contact with digesting food. Gastrointestinal cancer of the colon and rectum, or colorectal cancer (CRC) is a highly preventable disease with up to 43% of cases thought to be a result of two main disease determinants, diet and lifestyle [9,10]. Populations following a Western lifestyle show increased CRC incidence [11], while rural populations consuming high fiber plant foods show reduced CRC [12]. Mechanisms for chemopreventive activity of whole grains and legumes are likely related to the high-fiber content of both foods, but the association between fiber intake and overall CRC risk has proved inconsistent [7,13-15]. To date the actual mechanisms by which whole grain and legume consumption promotes human health remain largely uncharacterized. The current research seeks to clarify part of this knowledge gap.

Health benefits of fiber consumption

Dietary fiber plays an important role in human health. Soluble fiber exerts cholesterol lowering effects and insoluble fiber helps decrease gastrointestinal transit time [16]. By increasing intestinal transit time, viscosity and bulking, fiber lessens time of contact with colon tissues and concentration of potential carcinogens [17,18]. High fiber diets are recommended for CRC prevention, but the relationship of fiber intake to CRC occurrence has proved inconsistent

[7,13-15]. The largest meta-analysis on this relationship to date included more than 14,500 cases and almost 2 million participants and suggested a 10% reduction in risk for every 10 grams of fiber consumed daily [19]. Previously, chemopreventive effects associated with fiber consumption were related to these physiochemical properties, but the fermentation of fiber by intestinal microbiota and its subsequent metabolites are now credited with increasing importance [20].

Anaerobic fermentation of non-digestible plant fibers energizes colonocytes and reduces inflammation via microbial short chain fatty acids (SCFA), byproducts of this process [21]. Gut fermentation of fiber to SCFA, particularly butyrate is one proposed mechanism for improved chemoprevention with increased fiber consumption [21]. Butyrate is the primary energy source for colonic epithelia, helps maintain intestinal barrier function and has anti-proliferative properties [22]. In addition, fiber-rich foods contain a variety of other phytochemicals that have anti-oxidant and anti-inflammatory properties. These properties are responsible for the potential chemopreventive activity of these compounds, which include polyphenols, carotenoids, tocopherols and lignans [23].

Increasing plant fiber can also indirectly decrease pro-carcinogenic microbial metabolites such as secondary bile acids and trimethylamine N-oxide (TMAO) by supplanting animal protein and fat in the diet [24]. These harmful metabolites may also be bound and excreted by dietary fiber thereby reducing exposure of colon cells to secondary bile acids [17,25]. The role of fiber relative to other phytonutrients in colorectal chemoprevention remains unclear. The research presented herein aims to evaluate changes in phytonutrients, including fiber, and their secondary byproducts present in human stool following supplementation with foods derived from whole grain rice or legumes.

Selection of two high-fiber foods to be used for the current research

The purpose of the current research was to investigate the potential to favorably alter the intestinal environment for improved health, particularly for prevention of colorectal cancer (CRC) with dietary change. Two high-fiber foods were chosen for supplementation, heat-stabilized rice bran (SRB) and cooked navy bean powder (NBP). SRB and NBP (*Phaseolus* vulgaris) were chosen for study, because they are high-fiber staple foods that provide a source of bioactive phytochemicals that possess CRC chemopreventive activities in cell and animal models. Despite both being high in fiber, research to date suggests that SRB and NBP do not have equivalent effects on gut microbiota. For example, whole grain brown rice flakes were previously shown to increase gut bacterial diversity, Firmicutes:Bacteroidetes ratio and abundance of organisms from the genus *Blautia* [26]. Also, rice bran consumption has been shown to increase beneficial *Lactobacillus spp.* in animal studies [27]. However, limited studies in animal models have identified few effects of dry bean consumption on gut microbiota [28,29].

Rice bran, whole grain rice, and cooked and raw bean seeds have been the subject of research for their 'prebiotic' activity [30,31]. Prebiotic foods are defined by their resistance to human digestion; thereby entering the colon and serving as substrates for beneficial commensal bacteria. Both soluble and insoluble fiber shapes the composition of intestinal microbiota and influences their role in human physical and mental health [32-34]. The colonic bacteria break down these substrates into beneficial short chain fatty acids (SCFA), which regulate cell replication and apoptosis and reduce inflammation [35]. Production of SCFA also acidifies the intestinal environment creating unfavorable conditions for the establishment of some genotoxin-producing microorganisms [17,25]. Genotoxins directly damage DNA by breaking strands, creating point mutations, and inducing other numerical and structural changes that deregulate

proper cell growth and function and may lead to cancer. Both rice bran and beans were previously shown to increase fecal concentrations of SCFA in animal or *in vitro* models [30,36]. In addition, polyphenols contained in SRB [6] can selectively alter the gut microbial community [37], and are transformed into antioxidant phenolic acids [17,38,39]. Microbial production of these beneficial metabolites may play an important role in the ability of these foods to prevent CRC.

Nutrient and phytochemical composition of stabilized rice bran (SRB)

Rice bran contains all the outer brown layers of the kernel including the seed coat, pericarp, nucellus, and a portion of embryo-surrounding aleurone (Fig 1.1). However, polished white rice is composed of the starchy endosperm, germ and also a portion of the aleurone layer. The outer brown layers contain high levels of phytonutrients, oil, proteins and minerals important to human health while the inner white layers are predominated by carbohydrate [40]. SRB is a potential source for B vitamins and iron as well as vitamin E and some minerals [40]. In addition, SRB is an important source of macronutrients, soluble and insoluble dietary fibers, and small bioactive molecules [40]. Macronutrient breakdown is approximately 20% lipid, 15% protein, and 50% carbohydrate [41]. The lipid fraction contains monounsaturated fatty acid, primarily as oleic acid; also polyunsaturated fatty acid, primarily as linoleic acid. Saturated fatty acids, largely palmitic and stearic acids, are relatively minor making up approximately 18-29% of the lipid fraction [42]. Proteins contain a large amount of branched-chain amino acids including: leucine, isoleucine and valine. However, phenylalanine and lysine are also present in relatively high amounts and four other essential amino acids including histidine, methionine, threonine, and tryptophan are present in measurable amounts. More than half (54%) of the

carbohydrate fraction is composed of dietary and crude fiber, 1% sugars, and the remaining portion starches [41]. Total dietary fiber makes up 20% of SRB with only 2-3% as soluble fiber [43]. Compared to other grains (such as wheat, corn, or oat), more total SRB fiber is insoluble [16].

In addition to macronutrients, SRB is a source of important micronutrients and bioactive compounds. In addition to the B and E vitamins previously mentioned, these include: phenolic and cinnamic acids, anthocyanins and flavonoids, and phytosterols (plant-derived steroidal compounds). Phytosterols include γ-oryzanol, β-sitosterol ferulate, tocopherols and tocotrienols. Antioxidant properties can be attributed to anthocyanins, phenolic acid, and γ-oryzanol contents. Both phytosterols and fiber components of SRB contribute to improved blood lipid profiles [42]. Chemopreventive activity is attributed to over fifteen SRB components [6]. These include cellulose, hemicellulose, arabinoxylan and β-glucans found in dietary fiber in addition to saponin, squalene, tocotrienol, tocopherol and β-sitosterol found in the lipid fraction. Caffeic acid, ferulic acid and ferulic acid esters, all phenolics and from the lipid fraction of SRB, are also chemopreventive. Tricin, a flavonoid, is both cancer-preventive and free-radical scavenging. Phytic acid is also chemopreventive, but has the disadvantage of binding to iron and zinc in the digestive tract and preventing their absorption. In vivo studies with SRB, fermented SRB and rice bran extracts have shown chemopreventive activity for multiple cancer types including: colon, breast, lung, liver, and melanoma/skin cancers. A variety of mechanisms are proposed including cell anti-proliferation, tumor anti-proliferation, tumor-suppression and anti-inflammatory activity [6].

One issue of particular health concern related to rice consumption, in general, is arsenic content. Arsenic is a toxic metal that bio-accumulates in human and animal tissue and in some

plants, specifically rice. Arsenic content of rice varies according to region of growth and is dependent on the arsenic concentration of the growing soil and irrigation water with known areas of high arsenic in the US south and in Bangladesh [44,45]. Arsenic accumulates to the highest concentration in the hull and bran making arsenic levels of particular concern for SRB consumption [45,46]. However, rice grown in areas where soil and water do not contain arsenic, does not concentrate arsenic into tissue and should be safer to consume.

Heat stabilization of rice bran

While white rice is the culturally preferred form for rice consumption, substantial nutrition is lost in its production from brown rice. To produce white rice, harvested rice kernels are broken into these component parts: hull (non-nutritional), polished white rice, and rice bran [40]. The primary goal in rice milling is to maximize both yield and quality while producing polished white rice from brown rice. Quality is improved by removing the least amount of hull and bran possible, while also minimizing broken kernels and foreign matter [47]. Yield and value from the rice crop increases with increasing sophistication of the milling technology. In many remote and poorer areas, pestles and mortars or small single pass rice mills are still the most commonly used tools. In other countries, all of the rice is milled in larger sophisticated commercial mills.

Rice bran is a by-product of this milling process, more than 90% of which is currently sold cheaply as animal feed due to concerns about rancidity [42]. Rancidity results from bacterial lipases activated during milling that hydrolyze the lipid portion of rice bran into glycerol and fatty acid components. Once hydrolyzed, the fatty rice bran becomes rancid resulting in a highly

unpalatable smell and bitter taste [40]. This process is quite rapid, happening within six hours of milling [41].

Rice bran holds huge potential as a nutritive human food and is worthy of investment in stabilizing technologies. Many methods exist for stabilizing rice bran against rancidity, which provide potential for utilizing rice bran as a value-added health food product [41]. One option is heat stabilization, which can be accomplished via dry toasting, sun-drying, or steaming. The disadvantage to this approach is that it may destroy antioxidants found in bran. Cold storage can also prevent or delay rancidity and protects antioxidant activity. Ohmic heating is a newer technology, which courses electric current through the finished bran product, deactivating bacterial lipases, but also preserving most antioxidant activity [41]. Access to these stabilizing technologies is a barrier to using SRB for food in developing countries. Based on current estimates of rice production, there are roughly 66 to 74 million tons of bran that can be made available for human consumption. However, an important aspect of utilizing SRB as a human food source will be either making them more accessible, or developing more accessible alternatives for stabilization.

Nutrient and phytochemical composition of cooked navy bean powder (NBP)

The NBP utilized for this research was supplied by ADM (Archer Daniels Midland) Edible Bean Specialties, Inc. (Decatur, IL). The powder form was created by washing, soaking, and cooking whole navy beans and then grinding and dehydrating them to create a powder form [48]. NBP contains folate, Vitamins A and C and minerals including potassium, calcium, iron, magnesium, zinc, copper, and magnesium. Macronutrient breakdown is approximately 3% lipid, 24% protein, and 64% carbohydrate [48]. The lipid fraction is predominately polyunsaturated

fatty acids, but also contains monounsaturated and saturated fatty acids. Predominant amino acids include: leucine, lysine and phenylalanine. However, isoleucine, valine and threonine are also present in relatively high amounts. About one quarter of the carbohydrate fraction is composed of dietary fiber, 8.5% sugars, and the remaining portion starches [48]. Total dietary fiber makes up 26% of NBP with nearly 9% in the form of soluble fiber [48]. Compared to SRB, more total NBP fiber is soluble.

NBP is also a source of several bioactive compounds from similar chemical classes as those found in SRB including fiber, polyphenols, and phytic acid. Polyphenols found in *P. vulgaris* include tannins, lignans, and phytoestrogens (plant-derived estrogen receptor agonists). Phytoestrogens include the isoflavones, genistein and daidzein, which occur at much higher concentrations in soybeans relative to common beans, such as navy beans. Phytoestrogens are similar in chemical structure to estrogen and may prevent a number of hormone-influenced conditions including cancer, menopausal symptoms, CVD, and osteoporosis [49]. Lignans are components of plant cell walls and are similar in structure and potential activities to phytoestrogens [49]. Navy beans have a high antioxidant activity [50] possibly due to containing tannins, but do not contain detectable amounts of anti-oxidant flavonoids such as anthocyanins, quercetin glycosides and protoanthocyanidins found in other beans [51,52]. Phenolic compounds in common bean are attributed with anti-mutagenic effects [53,54] and consumption of navy beans was associated with reduced azoxymethane-induced CRC in rats [55]. As mentioned with SRB, phytic acid is also chemopreventive, but binds iron and zinc and prevents absorption. Furthermore, reduced mortality from CRC may be achieved by increasing consumption of beans [56,57]. Fermentation of dry bean insoluble fibers to SCFA has also been proposed to contribute to anti-inflammatory and anti-tumorigenic properties of dry beans [58].

Gut microbiota, diet and disease

Some research suggests that changes in diet alter gut microbiota and their metabolism as quickly as 24 hours [59]. Therefore, the potential of high-fiber foods like SRB and NBP to alter the gut microbial community via a prebiotic effect provides an important research opportunity. A growing body of research evidence connects gut microbial composition to multiple disease pathologies including cancer, obesity, type 2 diabetes, Celiac disease and inflammatory bowel disease [60]. CRC represents a unique opportunity for prevention studies because CRC survivors suffer from a high rate of recurrence that has been correlated to dietary habits, such as low fiber and antioxidant intake and high fat intake [61,62]. Furthermore, multiple studies report different gut microbiota composition in individuals diagnosed with colorectal cancer (CRC) versus healthy individuals [63-66]. Given that altered gut microbiota are associated with the CRC disease condition [67], beneficially altering this community and its associated metabolites shows promising potential for preventing CRC.

Specific gut microbiota can play a role in either promotion or prevention of CRC by modulation of the inflammatory process through close contact with host colonic mucosa [68]. When the gut microbial community exists in a state of balance where a diverse set of species is evenly represented, it helps to sustain intestinal health. However, if this balance is disrupted it can shift to a disease-promoting state of dysbiosis. Inflammatory processes can induce changes in the gut environment that favor the survival of specific microbial groups. In some cases these organisms further remodel the intestinal environment in a way that creates vulnerability to invasion by pathogenic organisms. Pathogens can amplify the inflammatory state by producing toxins or by feeding on and degrading the protective mucosal lining. The interaction between the

gut microbial community and host mucosa and its role in development of cancers, such as CRC, is explored in more detail in Chapter 2 of this work.

It's unclear to what extent dietary change may be able to rebalance a dysbiotic gut community or its metabolic processes, but these microorganisms and their metabolites have a significant influence on human health [22, 23]. Health benefits provided by diets rich in high-fiber whole grains and legumes may be partly due to beneficial changes to gut microbiota and their metabolites. Therefore, the current research seeks to clarify whether it may also be possible to use these foods as a preventive method to reverse the process of microbial dysbiosis and reinforce a healthy intestinal environment.

Research Objectives

Due to a lack of clarity regarding the chemopreventive mechanism of high fiber foods, the relative importance of whole grains, legumes, fruits and vegetables in high fiber diets remains unclear. By elucidating the mechanisms via which these foods provide CRC chemopreventive effects, better recommendations for dietary prevention and control of CRC can be provided. The hypothesis for this research was that gut commensal fermentation of two high fiber foods (SRB and NBP) plays an important role in CRC chemoprevention through the generation of beneficial metabolites. Three studies are included as part of this work: Study 1 (Chapter 3) compares stool microbiota and metabolites of healthy adults versus those diagnosed with CRC, Studies 2 and 3 (Chapters 4 and 5) investigate potential for dietary interventions with SRB or NBP to modulate stool microbiota and metabolites. Study 2 was conducted in adults with no history of CRC, while Study 3 participants are CRC survivors.

The research aim of Study 1 (Chapter 3) was to identify differences in stool metabolites and their associations with specific gut microbiota in healthy individuals compared to those with CRC. Stool metabolites were quantified and identified using non-targeted gas chromatography mass spectrometry (GC-MS) and stool bacteria were quantified and identified using 454 pyrosequencing along with appropriate bioinformatics workflows. Stool bacteria that differed with CRC were integrated with metabolite data in order to identify potential relationships between metabolites and the producing gut commensal. Stool metabolites provide an important measurable endpoint of gut microbial metabolism and assessing them was important for determining which gut microbial activities may be enriched or reduced with CRC. These data provided a foundation by which to compare outcomes of two pilot dietary interventions and hypothesize potential for prevention of CRC due to alterations in gut microbiota and their metabolites.

Study 2, the first pilot dietary intervention, was conducted in healthy individuals supplemented with SRB, NBP or macronutrient matched control diets for 28 days. First, this study aimed to confirm that SRB and NBP, at the administered dosage, are well tolerated and did not unduly disrupt gut eubiosis in a healthy population. Second, specific changes in gut microbiota and their metabolites with SRB and NBP supplementation were measured after 2 and 4 weeks. Non-targeted GC-MS was utilized for broad-spectrum stool metabolite analysis. This platform is most effective for detecting small, polar molecules such as amino acids and organic acids. GC-MS was also utilized to specifically quantify SCFA, due to their known importance to gut health. No significant changes to gut microbiota or metabolites were identified with NBP supplementation; therefore the results of that investigation are not included in Chapter 3. The

SRB data were compared to the existing literature base to postulate potential effects of these observed changes on intestinal health.

Study 3 is a similar pilot dietary intervention, except study participants were individuals previously diagnosed with CRC and more than 4 months post cancer treatment (e.g. chemotherapy or radiation). As in Study 2, participants were supplemented with SRB, NBP or macronutrient matched control meals for 28 days. Again, tolerance of SRB and NBP were confirmed and specific changes in gut microbiota and their metabolites were measured after 2 and 4 weeks. However, rather than utilizing a non-targeted metabolomics approach, metabolites were targeted based upon results from study 2 including: SCFA, bile acids, and microbial metabolites of phenolic compounds such as benzoic acid, 3,4-dihydrophenylacetic acid, phenylacetic acid, 3-phenylpropionic acid, 3-hydroxyphenylacetic acid, and 4-hydroxy-3-methoxycinnamic. In addition, predictive metagenomics was utilized to postulate potential functions of gut microbiota that were enriched with SRB supplementation. These combined data were compared to the existing literature base to postulate potential CRC chemopreventive effects in the intestinal environment.

These studies examine the influence of gut microbiota and their metabolism of dietary components in SRB and NBP on intestinal health, with an emphasis on potential mechanisms related to CRC prevention and control. Increasing plant foods, especially those high in fiber, is recommended for CRC prevention and to support intestinal health. However, much remains to be learned about the mechanism by which this health improvement occurs. By understanding gut fermentation of plant fibers, more effective dietary therapies can be developed for CRC prevention and overall intestinal health. In addition, investigations into mechanisms of gut microbial metabolism of dietary components will make it possible to provide more personalized

recommendations and therapies in the future based upon individual genetics and baseline gut microbial composition.

Figures

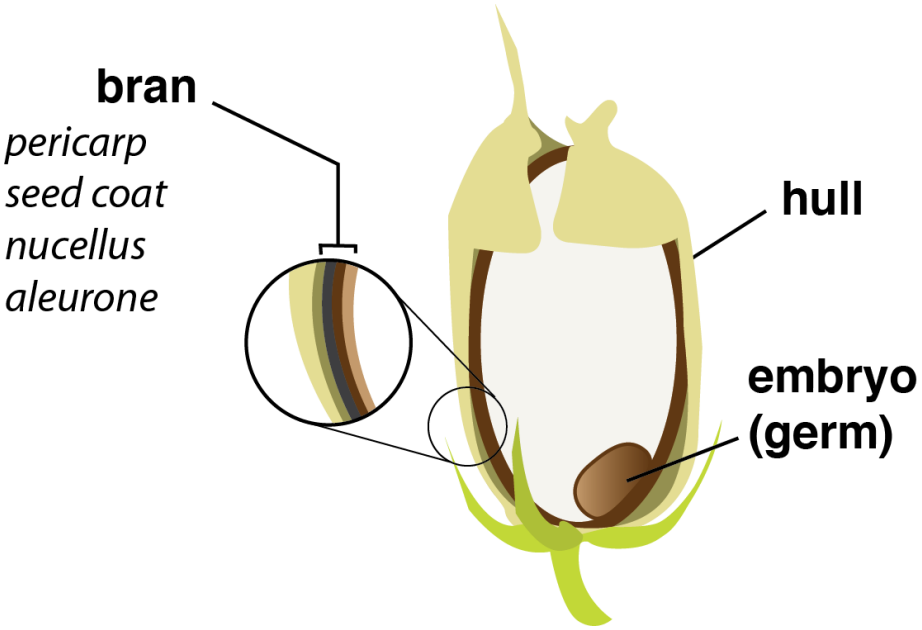


Figure 1.1. Structure of the rice grain

CHAPTER 2: SPECIFIC TOPIC REVIEW
CANCER-PROMOTING EFFECTS OF MICROBIAL DYSBIOSIS[12]

Summary

Humans depend upon our commensal bacteria for nutritive, immune-modulating and metabolic contributions to maintenance of health. However, this commensal community exists in careful balance that, if disrupted, enters dysbiosis; which has been shown to contribute to the etiology of colon, gastric, esophageal, pancreatic, laryngeal, breast and gallbladder carcinomas. This etiology is closely tied to host inflammation, which causes and is aggravated by microbial dysbiosis while increasing vulnerability to pathogens. Advances in sequencing technology have increased our ability to catalog microbial species associated with various cancer types across the body. However, defining microbial biomarkers as cancer predictors presents multiple challenges and existing studies identifying cancer-associated bacteria have reported inconsistent outcomes. Combining metabolites and microbiome analyses can help elucidate interactions between gut

[1] A modified version of this chapter is published as Sheflin, A.M., Whitney, A.K. and Weir, T.L., 2014. Cancer-promoting effects of microbial dysbiosis. *Current oncology reports*, 16(10), pp.1-9. *The final publication is available at Springer via http://dx.doi.org/ 10.1007/s11912-014-0406-0*

[2] The aim of this work was to conduct a literature review on the role of gut microbial dysbiosis in the development of various forms of cancer. This discussion provides background for interpretation of potential chemopreventive effects that could occur as a consequence of changes to gut microbial communities and their metabolites observed in other aims.

This chapter includes the complete published manuscript for this literature review titled *Cancer-Promoting Effects of Microbial Dysbiosis* (Amy M. Sheflin, Alyssa K. Whitney, Tiffany L.Weir, Journal of Oncology Reports, 2014). My contributions to this publication included writing the abstract and four sections: *Introduction, Proposed mechanisms for microbiome involvement in colorectal cancer, Role of microbial metabolites in cancer development and progression, and Conclusion.* I also conceptualized and created Figure 2.1, which is a visual summary of the important concepts in the paper. Finally, I edited and compiled all sections together into a cohesive manuscript prior to submitting.

Table and figure numbers have been modified to reflect that they are specific to this chapter, e.g. Figure 1 is now Figure 2.1. This article is reproduced with permission, and only minimal modifications were made to meet formatting requirements. No other modifications were made, as per the licensing agreement.

microbiota, metabolism and the host. Ultimately, understanding how gut dysbiosis impacts host response and inflammation will be critical to creating an accurate picture of the role of the microbiome in cancer.

Introduction

The relationship between specific pathogenic bacteria and human carcinogenesis has been the subject of extensive investigation. Historically, most of this research has focused on individual pathogens, such as *Helicobacter pylori,* and their potential to initiate and perpetuate disease. Previous research focus was on the disease process rather than beneficial gut-microbe interactions. More recently, extensive research supports commensal bacteria playing a role in protection of host health via nutritive, immune-modulating and metabolic processes [69,70]. In addition, the more holistic approach of characterizing entire communities of gut bacteria and their interactions is now possible through use of high-throughput DNA sequencing technology. Characterization of the gut microbiome as a whole has furthered our understanding of intestinal microbial ecology to include community-level functions and changes. In healthy individuals, the gut microbiome functions as a symbiont that can offer protection from invading pathogens and prevent tumorigenesis [71]. However, this commensal community exists in careful balance that, if disrupted, enters dysbiosis and contributes to host disease processes, including cancer [68,72-74]. While recent findings still support individual microorganisms influencing carcinogenesis, greater emphasis is on microbial dysbiosis and its larger role in cancer initiation and progression. The focus of this review is on gut microbial community dynamics that shift state from symbiosis to dysbiosis and the subsequent host immune and pathogen response, which drastically alters initiation and progression of multiple types of cancer.

Proposed mechanisms for microbiome involvement in colorectal cancer

Multiple studies report different gut microbiome composition in individuals diagnosed with colorectal cancer (CRC) versus healthy individuals [63-66]. In fact, gut microbiota can play a role in either promotion or prevention of CRC, often through modulation of the inflammatory process due to close contact with host colonic mucosa [68]. For example, chemically induced injury and proliferation induced by azoxymethane (AOM) and dextran sulfate sodium (DSS) was enhanced in germ free mice, which lack protective commensals. In addition, tumor development in the germ-free mice resulted in significantly more and larger tumors compared to specific pathogen free mice [75]. Balance of the gut microbial community, or eubiosis, can be disrupted by an inflammatory environment in the host. For example, host inflammation may influence microbiota composition through generation of specific metabolites such as nitric oxide synthase (NOS2). Nitrate provides a unique energy source for facultative anaerobic bacteria allowing them to outcompete bacteria that cannot utilize nitrates [76], disrupting balance of the gut microbiome and resulting in dysbiosis. Pro-inflammatory responses can also compromise barrier and immune function to allow bacterial translocation through intestinal tight junctions and intensify the inflammatory response [77].

How inflammation interacts with the gut microbiome to influence CRC has been recently synthesized in several hypotheses that summarize our understanding of the interactions to date (Figure 2.1). The 'alpha-bug' hypothesis suggests that a keystone pathogen species, such as Enterotoxigenic *B. fragilis* (ETBF), remodels the colonic microbiota to promote CRC, possibly via IL-17 and T_H17 cell-mediated inflammation. This process could also be initiated by microbial-independent host-mediated inflammation and may be blocked by beneficial commensal microbiota [78]. Similarly, the bacterial driver-passenger model suggests that 'driver'

bacteria, such as ETBF, cause or aggravate inflammation and produce genotoxins that lead to cell proliferation and mutations. Subsequently, an adenoma forms and is colonized by 'passenger' bacteria such as *Fusobacterium spp.* that encourage tumor progression [79]. Following tumor formation, the intestinal barrier is damaged by the continual inflammation and allows bacteria access to tumor tissue. These bacteria and their metabolites stimulate additional inflammatory signals, including IL-17 cytokines, promoting cancer progression [80]. Inflammatory signals may also stimulate macrophages, via induction to an M1 phenotype, to produce chromosome-breaking factors through a bystander effect, damaging DNA and inducing chromosomal instability in neighboring cells [71]. Likely CRC initiation and progression is engendered by aspects of each of these models.

Once bacteria translocate beyond a damaged intestinal epithelium, the host immune system responds with activation of multiple pattern recognition receptors (PRRs) PRRs important to the CRC process include membrane-bound Toll-like receptors (TLRs) and cytoplasmic NOD-like receptors (NLRs) [73]. Specifically, TLR2 and TLR4 have been shown to be important to tumor formation in murine models and recent associations between human genetic polymorphisms in TLR2 and TLR4 and CRC risk support a role in humans [81]. Furthermore, activation of nuclear factor (NF)-κB plays a role in CRC tumor initiation by enhancing both cytokines [72,82] and Wnt-signaling, which can convert intestinal epithelial nonstem cells into tumor-initiating cells [83]. The role of NF-κB in CRC is complex and involves additional signaling pathways which have recently been extensively reviewed [84]. Alternatively, in colitis-associated CRC, TLR signaling in tumor-associated fibroblasts initiates an inflammatory cascade independent of NF-κB via epiregulin (EREG). EREG stimulates the extracellular signal-regulated kinase (ERK) pathway, which encourages tumor proliferation [85].

Two NLRs are associated with CRC risk: NOD2, which is activated by the bacterial peptidoglycan, muramyl dipeptide; and NOD-, LRR-, and pyrin domain-containing 6 (NLRP6). With NOD2 deficiency, dysbiosis alone was sufficient for CRC development in mice [86]. However, recent research by Shanahan et al. reveals that NOD2-associated dysbiosis can be overcome by co-housing NOD2 mutants with wild type mice [87]. Future research is necessary to clarify the role of NOD2 and NLRP6 in gut microbial regulation. However, the role of bacterial translocation across intestinal epithelia in activation of TLR and NLR and in promoting inflammation is strongly supported [68,73,88,89].

Also 'driving' the cancer initiative process are pathogens that have been shown to promote tumorigenesis via genotoxic effects including: *Escherichia coli, Enterococcus faecalis,* and *Bacteroides fragilis.* Pathogenic strains of *E. coli* generally belong to groups B2 and D and produce genotoxic virulence factors, called cyclomodulins. Cyclomodulins can modulate cellular differentiation, apoptosis and proliferation [90] and include colibactin, cytotoxic necrotizing factor (CNF) and cytolethal distending toxin (CDT). Group B2 *E. coli* that produce cyclomodulins are highly prevalent in colonic mucosa of CRC patients [91]. *E. faecalis* indirectly increases genotoxin production in the form of DNA damaging reactive oxygen species (ROS) and reactive nitrogen species (RNS) by inducing an M1 phenotype in host macrophages [71]. ETBF releases fragilysin (also known as BST), a toxic virulence factor that induces DNA damage *in vivo* [92]. All of these organisms have also been shown to play a role in carcinogenesis via induction of inflammatory pathways [74]. In addition, *Fusobacterium* was recently associated with an upregulation of NF-κB–driven inflammatory genes and was identified as being enriched in colonic tumors [93]. While specific organisms exert these genotoxic effects, the effects are made possible and intensified through a prior state of dysbiosis.

Microorganisms associated with tumor occurrence and formation in CRC

A major goal of the Human Microbiome Project has been to define a "core" microbiome that could be useful in identifying deviations from a normal, healthy state. While the identification of a healthy core intestinal microbiome has remained elusive, numerous comparative studies have begun to reveal the relationship of the microbial community to CRC. The importance of the microbiome in tumor initiation and development has been elegantly demonstrated in murine models. Transfer of the microbiota from tumor bearing mice induces tumor formation in healthy animals [66] and mice with a genetic predisposition to develop CRC are spared when treated with antibiotics [94]. Retrospective human cohort studies encompass a range of sample types and populations, addressing questions related to global differences in the microbiome of healthy individuals relative to those afflicted with CRC or adenomatous polyps, and differences in the intestinal microclimates between healthy tissue and tumor tissue of an affected individual. Taken together, these studies are beginning to define a CRC-associated microbiome.

Although no bacteria have consistently been associated with CRC across all studies, the Gram-negative oral commensal *Fusobacterium nucleatum* has been most strongly linked to CRC. Several studies examining the colon tumor microenvironment by comparing tumor tissue to adjacent healthy tissue reported an overabundance of *Fusobacterium* associated with tumors [63,93,95]. A Chinese study reported a trend for increased *Fusobacterium* in tumor tissue relative to matched controls, but failed to achieve significance, which may be a result of the small study size (n=8), but could also indicate that *Fusobacterium* association with CRC is not consistent across different ethnicities [96]. Additional studies have confirmed that *Fusobacterium* spp. are enriched in pre-cancerous adenomas, particularly those displaying high

grade dysplasia [93,97]. Kostic et al. also reported higher stool levels of *Fusobacterium* in adenoma and CRC patients compared to healthy controls [93]. They also observed that ApcMin/+ mice infected with *F. nucleatum* had increased tumor multiplicity and selective recruitment of tumor-promoting myeloid cells. Activation of β-catenin signaling, which regulates inflammatory and oncogenic responses via binding of the FadA adhesin produced by *F. nucleatum* to E-cadherin in host membranes provides further evidence for the role of *Fusobacterium* as a driver of CRC initiation and progression [98].

Although it is known that mucosa adherent bacteria differ significantly from those found in the intestinal lumen, the identification of a CRC-associated stool microbiota is appealing for diagnostic and prognostic purposes. Unfortunately, there appears to be little consensus in the existing published literature of specific bacterial associations and even more general measures such as bacterial community diversity do not appear to consistently predict CRC. Sobhani et al. reported no differences in bacterial community diversity between case and control stool samples, but did note enrichment in *Bacteroides/Prevotella* in CRC stool samples, which was corroborated in mucosa samples from tissue biopsies [64]. They also reported depletion of *Bifidobacterium longan*, *Clostridium clostridioforme*, and *Ruminococcus* species. Another study reported higher levels of *Akkermansia muciniphila* and *Citrobacter farmeri* in CRC cases, and decreased butyrate-producing species such as *Ruminococcus* and *Roseburia* relative to controls [65]. *Akkermansia* is a common commensal in the intestines of humans and its depletion was previously associated with Crohn's disease and IBD [99]; however, it was demonstrated to be important in CRC tumor development in a murine model [66]. In the largest study to date examining stool microbes, a decrease in the microbial diversity of CRC cases was observed, as well as decreased *Clostridium* species [100]. This study also reported higher *Fusobacterium*

present in stool samples from CRC cases, suggesting possible utility of stool in reflecting mucosa levels of this tumor-associated bacterium. However, the composition of stool microbial communities appears to be a poor predictor of CRC presence based on current knowledge, and more large cohort studies are needed before effective diagnostic or prognostic tests can be developed using bacterial biomarkers in stool samples.

Microbiome Involvement in Gastric and Esophageal Cancers

Stomach and esophageal linings come in close contact with microbiota and recent evidence supports that the microbiome also influences these cancers. The longest-known and most extensively characterized association between these cancers and a gut microbe is with *H. pylori* infection. Mongolian gerbils, whose gastric system more closely resembles humans than the widely implemented mouse models, showed that 37% of *H. pylori* infected animals developed adenocarcinomas while no tumor development occurred in uninfected controls [101]. More recent work with this animal model suggests that long-term *H. pylori* infection disrupts the gut microbial community. *H. pylori* negative gerbils were observed to have decreased abundance of *Bifidobacterium* spp. , *C. coccoides* group and *C. leptum* subgroup but a higher abundance of *Atopobium* cluster [102]. In addition, three lactobacillus species, *L. reuteri, L. johnsonii,* and *L. murinus* inhibit *H. pylori* growth *in vitro,* suggesting that some gut microbes may help prevent *H. pylori* infection [103].

Human studies comparing stomach microbiota in cancer patients and healthy controls indicate that microbes other than *H. pylori* must be present to facilitate mucosal movement toward gastric cancer development [104,105]. In fact, many people infected with *H. pylori* do not develop gastric cancer [105]. Aviles-Jimenez et al. noted decreases in *Porphyromonas,*

Meisseria, and *Streptococcus sinesis* and increased *Lactobacillus coleohominis, Pseudomonas* and Lachnospiraceae among gastric cancer patients [104]. The noted increase in *L. coleohominis,* a species previously thought to be beneficial, is supported by Dicksved et al., who measured an increase in terminal restriction fragments (TRFs) corresponding to *Lactobacilli* in gastric cancer patients' samples [105]. Further investigation of this phenomenon and of *H. pylori* interactions with the gut microbiome is required to better understand its role in the disease process.

Eradication of *H. pylori* has been shown to correlate with a decrease in incidence of gastric cancer [105]. Shin et al. showed a decrease in the methylation of the LOX tumor suppressor gene with eradication of *Helicobacter felis,* the murine equivalent of *H. pylori* [106]. A study by Cai et al. indicates that eradication therapy is most effective in restoring parietal cells and reducing dysplasia when *H. felis* infection duration is less than 6 months. Infections lasting longer than this time period, when dysplasia and metaplasia are more severe, resulted in only partial reversion of these lesions [107]. Results from human studies of *H. pylori* eradication for prevention of gastric cancers are conflicting and need to be conducted on larger cohorts with longer follow-ups in order to assess the effectiveness of this strategy for chemoprevention.

Several mechanisms have been proposed by which *H. pylori* induces development of gastric cancer. *Helicobacter pylori* infection increases cell proliferation, leading to the increased turnover of the gastric mucosa which could lead to a higher incidence of mutation and less time for DNA repair [101]. Mice lacking secretory phospholipase A2 (sPLA2), such as C57BL/6 the showed increased levels of apoptosis after oral infection with *H. felis* and expansion of aberrant gastric mucosa cell lineages; indicating that sPLA2 influences the response of gastric mucosa to *H. felis* infection [108]. Raf-kinase inhibitor protein (RKIP) regulates the cell cycle and apoptosis in the gastric mucosa. In infected mucosa, *H. pylori* phosphorylates RKIP, removing

apoptotic control and inducing proliferation by removing control of the cell cycle [109]. Another tumor suppressor gene, LOX, was shown by Shin et al. to be methylated in transgenic mice infected by *H. felis* [106]. The down-regulation of these tumor-suppressing proteins allows gastric adenocarcinoma to develop in the presence of *H. pylori* infection.

Helicobacter pylori infection has also been implicated in the development of esophageal cancer, but its role is unclear [110]. Anderson et al. showed an increase in seropositivity for *H. pylori* in junctional tumors, those involving the esophagus and gastric cardia. However, in tumors that do not involve the gastric cardia, *H. pylori* is associated with a lowered risk of tumor development [111]. More is known about the microbiome of reflux esophagitis and Barrett metaplasia, which are precursor states to esophageal cancer. In these conditions, dominance shifts from Gram-positive bacteria to mostly Gram-negative, suggesting that dysbiosis plays a role in the disease process [112]. It's likely that other microbes are also involved in tumor development in the esophagus. Cancerous esophageal tissue shows a higher prevalence of *Treponema denticola, Streptococcus mitis,* and *Streptococcus anginosus*, as compared to normal tissue. These pathogens induce inflammation by cytokines, possibly supporting tumor development [113].

Microbiome Involvement in Other Forms of Cancer

The microbiome has also been implicated in the etiology of pancreatic [114], laryngeal [115], and gallbladder [116] carcinoma. Farrell et al. noted significant shifts in oral microbial composition between healthy and pancreatic cancer groups. Among cancer groups, significant decreases were noted in *Neisseria elongata and Streptococcus mitis* (a pathogen also implicated in esophageal cancer [113]). These significant changes in oral microbiota with the development

of pancreatic cancer indicate potential for oral *N. elongate* and *S. mitis* to serve as biomarkers for pancreatic cancer occurrence [114].

In gallbladder cancer, *Salmonella* infection is shown to be of particular importance [116] . The gallbladder is a known reservoir of *Salmonella*, leading to increases in secondary bile acid concentrations, which linked to tumor promotion [117]. Sharma et al. showed an association between the typhoid carrier state and gallbladder cancer. In addition, bile culture-positivity is associated with increase in gallbladder carcinogenesis, especially positivity for the Vi antigen (a capsular antigen associated with *Salmonella*) [116]. These associations indicate the relevance of the microbiome in the etiology of multiple cancer types.

Viruses are also a component of the gut microbiome and can influence cancer risk. For example, DNA from Human Papillomavirus (HPV) is detected in almost all cervical cancers [118]. Extensive study indicates that viral antigens E6 and E7 contribute to the malignancy of HPV-induced cervical cancer [119]. However, estrogen is required for the development of cervical cancer from HPV infection. In mice and rats, 83% of HPV infected animals develop cervical cancer after estrogen treatment [120]. Estrogen treatment leads to increased transcription of viral antigens E6 and E7, contributing to cervical carcinogenesis [119]. In addition, the presence of estrogen receptor α (ERα) is necessary in the development of cervical cancer from HPV infection [121,122], as ERα knockout mice do not develop cervical cancer when infected with HPV [121]. As intestinal microbes affect circulating estrogen levels [123], these commensal organisms may be involved in the development of cervical cancer from HPV infection; however, further study is needed to support this link.

HPV, in conjunction with *H. pylori*, has also been implicated in laryngeal cancer [115,124]. Gong et al. associated a total of 15 additional genera with laryngeal carcinoma tissue,

with noted increases in *Fusobacterium, Prevotella,* and *Gemella.* *Fusobacterium* and *Prevotella* in particular are thought to be associated with the development of biofilms that stimulate an inflammatory response [115], leading to laryngeal cancer development [125]. While HPV and *H. pylori* are both involved in laryngeal cancer development, not much is currently known about how viral and bacterial members of the microbiome interact, an intriguing topic for future research.

Role of microbial metabolites in cancer development and progression

Changes in bacterial metabolism can modulate cancer risk and often accompany dysbiosis of the gut microbiome. Specific bacterial metabolites associated with increased CRC risk include: prostaglandin E2 [126] and multiple secondary bile acids (SBAs) [65]. Conversely, decreased CRC risk is associated with indole [127], anti-oxidants [126] and the anti-proliferative metabolites butyrate [65] and ursodeoxycholic acid [65]. Indole, a bacterial quorum-sensing molecule produced by catabolism of tryptophan, enhances barrier function of colonic epithelial cells *in vitro*. *In vivo* experiments suggest indole is a byproduct of gut microbial metabolism as indole is significantly lower in germ free mice compared to specific pathogen free mice. *In vivo* experiments also suggest that indole enhances function of both tight-junctions and adherens junctions in both germ-free and specific pathogen free mice [127]. Butyrate has known anti-tumorigenic and anti-proliferative effects due to its regulation of genes that inhibit cell proliferation and induce apoptosis via histone deacetylase (HDAC) inhibition [128]. Ursodeoxycholic acid (UDCA), a microbial metabolite of a primary bile acid, has been shown to prevent colorectal tumor development in animal and preclinical models [129]. UDCA has been administered in clinical trials as a chemopreventive agent and a systemic review of UDCA's

effect on the incidence or recurrence of CRC is currently underway [130]. However, some evidence also exists to suggest that UDCA may be pro-carcinogenic at higher doses [131].

Secondary bile acids (SBAs) such as deoxycholic (DCA) and lithocholic acid (LCA) are produced as products of microbial metabolism of primary bile acids produced by the host. The promotion of CRC by DCA and LCA and other SBAs has recently been extensively reviewed [132]. Recent evidence points to bacteria in Clostridium cluster IX as a possible source of increased DCA and cancer risk in obese mice [133]. DCA in particular was found to increase rapidly, with 24 hours, on an animal based diet and was linked to overgrowth of inflammation causing microorganisms associated with inflammatory bowel disease [59]. However, DCA also acts as a ligand of the FXR receptor [134], which has been shown to reduce liver and intestinal tumor growth and metastasis [135]. Similarly, LCA may prevent DNA damage, and therefore tumorigenesis, through stimulation of xenobiotic metabolism and excretion [136]. While LCA and DCA are predominantly characterized as promoting CRC, future research in this area may reveal a more complex role for these metabolites in the CRC process.

Extensive study indicates a role of intestinal microbes in the metabolism of dietary estrogens. In patients treated with ampicillin, fecal excretion of estrogen metabolites increases, indicating that re-absorption into the bloodstream is reduced with diminished intestinal microflora [137]. Adding further support to the involvement of intestinal microflora in estrogen metabolism, fecal microbes are shown to carry out oxidation and reduction reactions on estrogens and can shift intestinal concentrations of estrone and estradiol [123]. Although no definite link has been observed between intestinal microflora estrogen metabolism and cancer development, it is reasonable to anticipate the existence of such a mechanism.

Definitive linkage between estrogen levels and breast cancer development has been shown [138]. In rat models, implanted estrogen leads to cyst formation in mammary tissue [139]. In addition, the presence of anti-estrogen antibodies- decreasing estrogen concentrations- delays the onset and growth of mammary tumors in rats and mice [140]. Specifically, 16α hydroxylation of estrogen, a reaction shown to be carried out by the intestinal microflora [123] is associated with an increase in risk for the development of breast cancer [141]. Considering these results and the similarity between the etiology of colorectal cancer and breast cancer, Hill et al. hypothesized a link between breast cancer development and metabolism of estrogen by intestinal microflora [140].

Recent techniques combine analyses of changing metabolites and microorganisms in an effort to understand interactions between gut microbiota, metabolism and the host [65,142,143]. Further research in this area will deepen the mechanistic understanding of microbial metabolism in the cancer disease process.

Conclusion

The role of microorganisms in cancer initiation and progression can no longer be simply described as a pathogen-disease relationship. Evidence that our microbiome also functions to promote health and prevent disease by encouraging apoptosis and limiting proliferation and inflammation is growing. A microbiome in a state of balance helps to sustain human health, but as this balance is disrupted via inflammatory processes the community changes and becomes vulnerable to invasion by pathogenic organisms. If these pathogens successfully establish, then a disrupted state of dysbiosis occurs allowing for further inflammation and production of genotoxins and other carcinogenic microbial metabolites. In addition, dysbiosis was recently

hypothesized to contribute to the evolution of pathogens, which could potentially raise cancer risk [144].

However, as we begin to better understand the gradient of eubiosis to dysbiosis (Fig 1), we can develop methods to manipulate the gut microbiome to promote health. As an example, we already know that diet plays a large role in bacterial species of the microbiome, their metabolites and cancer risk. A recent study looked at rural Africans who exhibit significantly lower risk of CRC compared to African Americans. Rural Africans were shown to have increased *Prevotella* spp. and butyrate as compared to African Americans who had higher *Bacteroides* spp. and SBAs [142]. These differences may be a consequence of Rural Africans having higher resistant starch intake and African Americans having higher meat and fat intakes [12]. Dietary choices can affect cancer risk [142,143,145] and changing diet to potentially reduce risk is the exciting topic of much current study [146,147]. Diet represents just one example of how to apply our growing knowledge of gut microbiome dynamics toward health promotion and disease prevention. Other potential therapies to modulate the gut microbiome include fecal transplants [148], probiotics [149,150], exercise [151] and likely many more that we may have failed to mention. Future studies should focus on these therapies and their mechanisms to improve applications in a clinical setting.

Figures

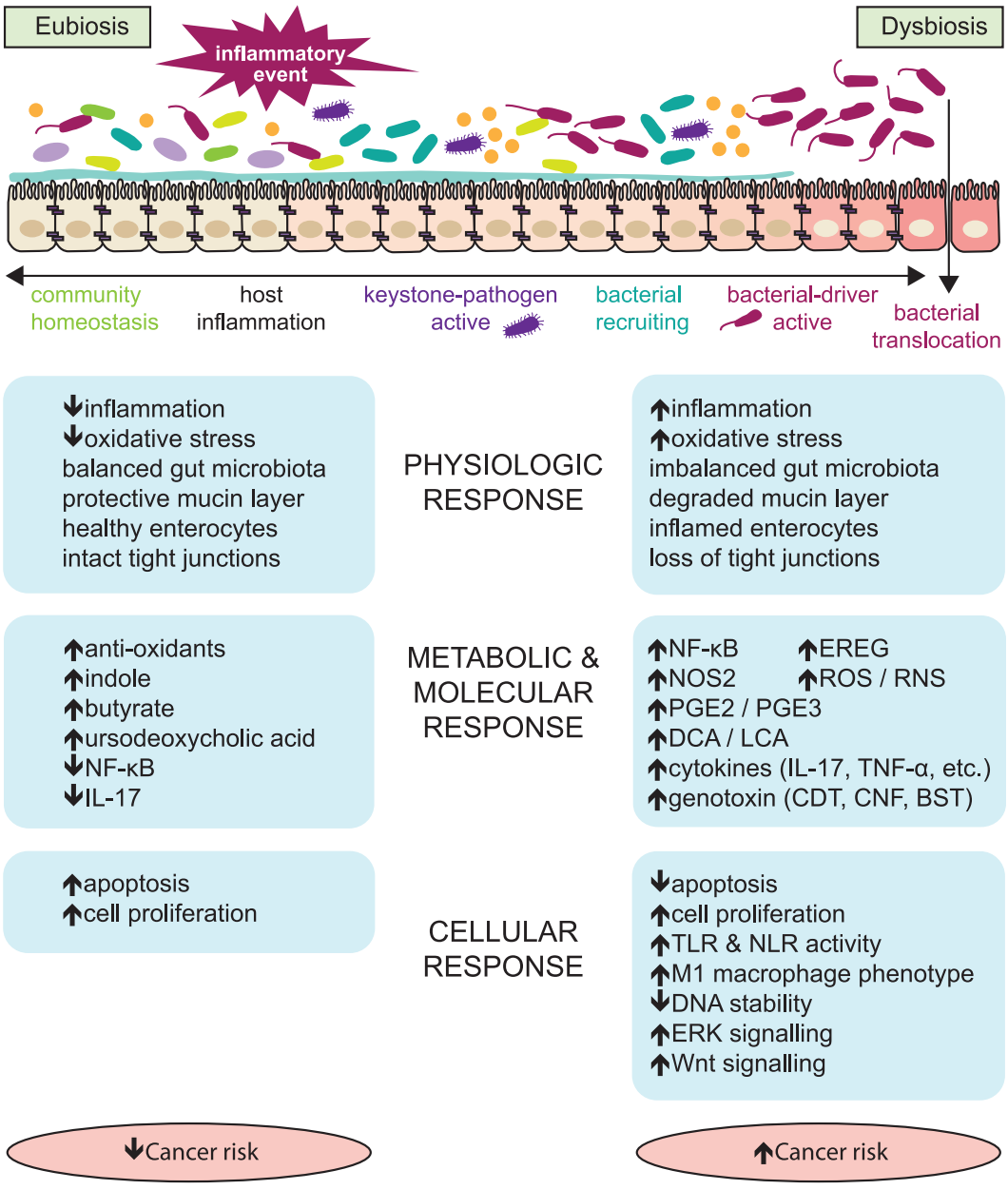


Figure 2.1. The progression of the gut microbial community from a state of balance (eubiosis) to imbalance (dysbiosis) is associated with physiologic, metabolic and cellular responses in the host that modulate cancer risk.

CHAPTER 3: STOOL MICROBIOME AND METABOLOME DIFFERENCES BETWEEN COLORECTAL CANCER PATIENTS AND HEALTHY ADULTS[34]

Summary

In this study we used stool profiling to identify intestinal bacteria and metabolites that are differentially represented in humans with colorectal cancer (CRC) compared to healthy controls to identify how microbial functions may influence CRC development. Stool samples were collected from healthy adults (n=10) and colorectal cancer patients (n=11) prior to colon resection surgery at the University of Colorado Health-Poudre Valley Hospital in Fort Collins, CO. The V4 region of the 16s rRNA gene was pyrosequenced and both short chain fatty acids and global stool metabolites were extracted and analyzed utilizing Gas Chromatography-Mass Spectrometry (GC-MS). There were no significant differences in the overall microbial

[3] A modified version of this chapter is published as Weir, T.L., Manter, D.K., Sheflin, A.M., Barnett, B.A., Heuberger, A.L. and Ryan, E.P., 2013. Stool microbiome and metabolome differences between colorectal cancer patients and healthy adults. *PloS one*, *8*(8), p.e70803. *The final publication and supporting material is available from PLOS at http://dx.doi.org/10.1371/journal.pone.0070803*

[4] The aim of this work was to compare stool microbiota and metabolites from individuals diagnosed with CRC to healthy individuals. These results give context for interpretation of microbiota and metabolites that are altered with dietary intervention with heat-stabilized rice bran and beans.

This chapter includes the complete published manuscript for this research titled *Stool microbiome and metabolome differences between colorectal cancer patients and healthy adults* (Weir, T.L., Manter, D.K., Sheflin, A.M., Barnett, B.A., Heuberger, A.L. and Ryan, E.P., *PloS one,* 2013). My contributions to this publication included the global metabolomics annotation, analysis, and interpretation and visual representations of this work are represented in Table 3.7, Figure 3.4 and Figure 3.5.

Table and figure numbers have been modified to reflect that they are specific to this chapter, e.g. Figure 1 is now Figure 3.1. In addition, figures were renumbered to incorporate supplemental material into the flow of the chapter. This article was published under open access and is reproduced under the creative commons license. Only minimal modifications were made to meet formatting requirements. No other modifications were made.

This work is licensed under a Creative Commons Attribution 4.0 International License. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited. To view a copy of this license, visit https://creativecommons.org/licenses/by/4.0/

community structure associated with the disease state, but several bacterial genera, particularly butyrate-producing species, were under-represented in the CRC samples, while a mucin-degrading species, *Akkermansia muciniphila*, was about 4-fold higher in CRC ($p<0.01$). Proportionately higher amounts of butyrate were seen in stool of healthy individuals while relative concentrations of acetate were higher in stools of CRC patients. GC-MS profiling revealed higher concentrations of amino acids in stool samples from CRC patients and higher poly and monounsaturated fatty acids and ursodeoxycholic acid, a conjugated bile acid in stool samples from healthy adults ($p<0.01$). Correlative analysis between the combined datasets revealed some potential relationships between stool metabolites and certain bacterial species. These associations could provide insight into microbial functions occurring in a cancer environment and will help direct future mechanistic studies. Using integrated "omics" approaches may prove a useful tool in identifying functional groups of gastrointestinal bacteria and their associated metabolites as novel therapeutic and chemopreventive targets.

Introduction

A healthy gastrointestinal system relies on a balanced commensal biota to regulate processes such as dietary energy harvest [152], metabolism of microbial and host derived chemicals [153], and immune modulation [154]. Accumulating evidence suggests that the presence of microbial pathogens or an imbalance in the native bacterial community contributes to the development of certain gastrointestinal cancers. A causal relationship between gastric cancer and *Helicobacter pylori* has been established [155], leading to the hypothesis that other host-associated organisms are involved in cancer etiology.

An association between colorectal cancer (CRC) and commensal bacteria has been suspected for decades. For example, *Streptococcus infantarius* (formerly *S. bovis*) became diagnostically important after it was recognized that bacteremia due to this organism was often associated with colorectal neoplastic disease [156,157] . However, early studies associating genera of bacteria with colon cancer risk were limited to culture-based methods that did not reflect the complexity of the gastrointestinal microbiota [158-160]. Development of high-throughput sequencing has facilitated detailed surveys of the gut microbiota, and a more thorough and complex colorectal cancer (CRC)-associated microbiome is emerging. Sobhani et al. [64] found that the Bacteroides/*Prevotella* group was over-represented in both stool and mucosa samples from individuals with colon cancer compared to their cancer-free counterparts. They also found that *Bifidobacterium longum*, *Clostridium clostridioforme*, and *Ruminococcus bromii* were underrepresented in samples from these individuals and concluded that a lack of correlation between tumor stage/size with the over-represented populations suggested a contributory role of the bacteria in tumor development. Two additional studies, published concurrently, examined the microbiota present in the tumor mucosa and adjacent healthy tissue of individuals with colon cancer and both studies revealed an overrepresentation of *Fusobacterium spp* [161,162], while others have revealed an abundance of *Coriobacteria* and other probiotic species [163,164].

The question remains whether over-representation of particular microbial species in stool and mucosal samples is indicative of a contributory role in the development of CRC or a consequence of the tumor environment. Although a causal role of intestinal biota in CRC development has not been demonstrated, there is evidence to suggest that induction of pro-inflammatory responses by commensals contribute to tumor initiation and development [64,164].

Production of genotoxins and DNA damaging superoxide radicals are also mechanisms by which commensals can contribute to CRC development [165]. Alternatively, it has been hypothesized that certain probiotic bacteria act as tumor foragers, taking advantage of an ecological niche created by the physiological and metabolic changes in the tumor microenvironment [164].

To clarify the role of intestinal biota in the development of CRC, it will be necessary to move beyond taxonomic over-representation and examine changes in the CRC associated microbiome in a more functional context. One important functional parameter is how commensal organisms contribute to the flux of metabolites and the breakdown of dietary components. Thus, metabonomics, the study of global changes in metabolites in response to biological stimuli [166], is being applied to identify and characterize the functional microbiome that drives metabolic changes associated with different diets, genotypes, and disease states [167-169]. Stool metabolite profiles have been validated as a means of assessing gut microbial activity [170] and the current study contributes to the growing list of gut microbes in the CRC microbiome, but also utilizes a metabonomics approach to identify potential microbiome-metabolome interactions.

Materials and Methods

Ethics statement.

All individuals provided written informed consent prior to participating in the study. All study protocols were approved by Colorado State University (Protocol numbers 10-1670H and 9-1520H) and Poudre Valley Hospital-University of Colorado Health System's Institutional Review Boards (Protocol numbers 10-1038 and 10-1006).

Sample collection and DNA extraction.

Stool samples were collected from healthy individuals (n=11) and recently diagnosed colon cancer patients (n=10) prior to surgery for colonic resection (Table 3.1-note: not all samples were subjected to all analyses. See Table 3.1 footnote). Exclusion criteria for all participants included use of antibiotics within two months of study participation, and regular use of NSAIDS, statins, or probiotics. Individuals that reported chronic bowel disorders or food allergies/dietary restrictions were also excluded from the study. Additional exclusion for CRC patients included chemotherapy or radiation treatments prior to surgery. Stool samples were provided for analyses prior to administration of any pre-operative antibiotics or bowel preparation. Samples were transported to the laboratory within 24 hours after collection by study participants. Stool samples were homogenized, and three subsamples were collected with sterile cotton swabs. DNA was extracted from all samples using MoBio Powersoil DNA extraction kits (MoBio, Carlsbad, CA) according to the manufacturer's instructions and stored at -20^{o}C prior to amplification steps.

Pyrosequencing analysis.

Amplification of the V4 region of the bacterial 16S rRNA gene was performed in triplicate using primers 515F and 806R labeled with 12-bp error correcting Golay barcodes [171]. Twenty reactions containing 5 Prime Hot Master Mix (5 Prime, Inc., Gaithersburg, MD) were amplified at 94^{o}C for 5 minutes followed by 35 cycles of 94^{o}C for 1 min, 63^{o}C for 1 min, and 72^{o}C for 1 min followed by a final extension at 72^{o}C for 10 minutes. Replicate PCR reactions from each sample were combined and gel purified using the GenElute Gel Extraction kit (Sigma-

Aldrich, St. Louis, MO), followed by an additional purification with AMpure beads (Beckman Coulter, Indianapolis, IN) and quantified with the PicoGreen DNA Assay (Invitrogen, Carlsbad, CA, USA) prior to library pooling. Pyrosequencing was performed under contract by the University of South Carolina's Engencore Sequencing Facility using a 454 Life Sciences GS FLX System with standard chemistry.

All sequence read editing and processing was performed with Mothur Ver. 1.25 [172] using the default settings unless otherwise noted. Briefly, sequence reads were (i) trimmed (bdiff=0, pdiff=0, qaverage=25, minlength=100, maxambig=0, maxhomop=10); (ii) aligned to the bacterial-subset SILVA alignment available at the Mothur website (http://www.mothur.org); (iii) filtered to remove vertical gaps; (iv) screened for potential chimeras using the uchime method; (v) classified using the Green Genes database (http://www.mothur.org) and the naïve Baysian classifier [173] embedded in Mothur. All sequences identified as chloroplast were removed; (vi) sequences were screened (optimize=minlength-end, criteria=95) and filtered (vertical=T, trump=.) so that all sequences covered the same genetic space; and (vii) all sequences were pre-clustered (diff =2) to remove potential pyrosequencing noise and clustered (calc=onegap, coutends=F, method=nearest) into OTUs [174]. To remove the effect of sample size on community composition metrics, sub-samples of 1250 reads were randomly selected from each stool sample. After clustering sequence reads into OTUs (i.e., nearest-neighbors at 3% genetic distance) or phylotypes (i.e., sequences matching a common genus in the Green Genes Database), the replicate sub-samples were averaged to yield a single community profile for each sample. Sample size independent values for alpha diversity community descriptors such as observed species richness (S_{obs}), Chao1 estimates of total species richness (S_{Chao}), Shannon's diversity (H') and evenness (E_H), and Simpson's diversity (1-D) and evenness (E_D) were

determined by fitting a 3-parameter exponential curve [$y = y0 + a(1\text{-}e^{-bx})$] to rarified parameters over a range of 100 to 1250 sequence reads, where the asymptotic maxima is equal to the sum of *y0* and *a*. Effective number of species were calculated as S_H=exp (H') for the Shannon's index and S_D=1/D for Simpson's. All sequence data is publicly available through the Sequence Read Archive (SRA) under study accession number ERP002217, which is available at the following link: http://www.ebi.ac.uk/ena/data/view/ERP002217.

Nontargeted Metabolite Profiling and Data Processing Methods.

One hundred milligrams of lyophilized stool sample were extracted two times with 1 ml of 3:2:2 isopropanol:acetonitrile:water spun at 14,000 rpm for 5 minutes and the supernatants were combined. The extract was dried using a speedvac, resuspended in 50 µL of pyridine containing 15 mg/mL of methoxyamine hydrochloride, incubated at 60ºC for 45 min, sonicated for 10 min, and incubated for an additional 45 min at 60ºC. Next, 50 µL of N-methyl-N-trimethylsilyltrifluoroacetamide with 1% trimethylchlorosilane (MSTFA + 1% TMCS, Thermo Scientific) was added and samples were incubated at 60 ºC for 30 min, centrifuged at 3000xg for 5 min, cooled to room temperature, and 80 µL of the supernatant was transferred to a 150 µL glass insert in a GC-MS autosampler vial. Metabolites were detected using a Trace GC Ultra coupled to a Thermo DSQ II (Thermo Scientific). Samples were injected in a 1:10 split ratio twice in discrete randomized blocks. Separation occurred using a 30 m TG-5MS column (Thermo Scientific, 0.25 mm i.d., 0.25 µm film thickness) with a 1.2 mL/min helium gas flow rate, and the program consisted of 80 ºC for 30 sec, a ramp of 15 ºC per min to 330 ºC, and an 8 min hold. Masses between 50-650 m/z were scanned at 5 scans/sec after electron impact ionization. For each sample, a matrix of molecular features as defined by retention time and mass

(m/z) was generated using XCMS software [175]. Features were normalized to total ion current, and the relative quantity of each molecular feature was determined by the mean area of the chromatographic peak among replicate injections (n=2). Molecular features were formed into peak groups using AMDIS software [176], and spectra were screened in the National Institute for Technology Standards (www.nist.gov) and Golm (http://gmd.mpimp-golm.mpg.de/) metabolite databases for identifications.

SCFA determination.

Stool samples were extracted for short chain fatty acids by mixing 1g of frozen feces with acidified water (pH 2.5) and sonicated for 10 min. Samples were centrifuged and filtered through 0.45uM nylon filters and stored at -80ºC prior to analysis. The samples were analyzed using a Trace GC Ultra coupled to a Thermo DSQ II scanning from m/z 50–300 at a rate of 5 scans/second in electron impact mode. Samples were injected at a 10:1 split ratio, and the inlet was held at 22°C and transfer line was held at 230°C. Separation was achieved on a 30m TG-WAX-A column (Thermo Scientific, 0.25 mm ID, 0.25 μm film thickness) using a temperature program of 100°C for 1 min, ramped at 8°C per minute to 180°C, held at 180°C for one minute, ramped to 200°C at 20°C/minute, and held at 200°C for 5 minutes. Helium carrier flow was held at 1.2 mL per minute. Peak areas were integrated by Thermo Quan software using selected ions for each of the short chain fatty acids, and areas were normalized to total signal.

Statistical Analysis.

Differences in bacterial phylotypes and global metabolites between samples from healthy individuals and colon cancer patients were determined using AMOVA and student's t-tests with

a significance cutoff of <0.01. Phylotypes and metabolites that were significantly different between groups were further refined by removing markers that had fewer than 25 total reads (bacteria) or borderline background signals (metabolites) or that were present in fewer than 3 individual samples. Short chain fatty acid concentrations were determined in two separate chromatographic runs, so a weighted mean was calculated for each quantified compound and statistical differences between stool samples from healthy individuals and colon cancer patients were determined using a mixed model ANOVA with experiment representing a random effect and disease status as a fixed effect (XLSTAT 2011.1, Addinsoft Corp, Paris, France). Correlations between metabolites and bacteria were determined using Pearson's r with a moderate correlation denoted by an $r \geq 0.50$ and a strong correlation denoted by an $r \geq 0.70$.

Results and Discussion

Alpha and beta diversity in stool biota.

Typical community descriptors of alpha diversity for molecular microbial data include actual and estimated OTU richness, and indices of population diversity and evenness. In systems where pathogens are introduced (e.g. *Helicobacter pylori)*, there are marked decreases in estimates of diversity and evenness [177] suggesting that these indices may be useful predictors of infection. We examined these parameters in stool samples from healthy individuals and those with CRC to see if they could be used as predictors of disease state. We observed no significant differences at the 3% genetic distance in the average diversity or evenness of stool microbial communities from healthy individuals compared to those with CRC (Table 3.2). The average coverage obtained from 1250 reads per sample was 84% and 86% in healthy and colon cancer samples respectively. The average effective diversity of each group suggested a trend toward

higher bacterial diversity in stool samples of healthy individuals ($S_H = 63$, $S_D = 20$) compared to those from CRC patients ($S_H = 46$, $S_D = 15$); however, the inter-individual variation was too great to achieve statistical significance. Based on these data, we suggest that alpha diversity descriptors of stool microbiota are not indicative of disease state in CRC; although a limitation of this study is that only stool samples and not tissue mucosa were analyzed. However, despite inherent differences in stool and mucosal microbial communities our findings are consistent with other published reports of total bacterial diversity and evenness estimates between CRC and healthy stool and tissue/mucosasamples [64].

This inter-individual variation was also apparent in estimates of beta diversity, where a low degree of similarity in overall microbial community composition between individuals was observed as determined using the unweighted Jaccard distance (J_{class}) to compare community membership (Figure 3.1A) and Yue and Clayton's [178] index (Q_{YC}) to compare community structures (Figure 3.1B). Because of this variation, no patterns in the overall community composition were noted between stool samples from CRC patients and healthy individuals.

Taxonomic differences between CRC and healthy stool samples.

The disease status of study participants did not drive overall community structure of the stool microbiota, and the composition and relative abundance of the major phyla were similar, although there was a non-significant trend towards higher Verrucomicrobia in samples from colon cancer patients (Figure 3.2). There were also higher levels of Synergetes in the cancer group, but this was driven by a single individual with an extremely high proportion of this phyla and was not representative of the entire sequenced cancer population. However, at the genus/species level there were a number of OTU's that were significantly under-represented in

the stool of colon cancer patients compared to healthy individuals (Table 3.3). These include several Gram-negative *Bacteroides* and *Prevotella spp*. that have previously been isolated from human stool, but are not well characterized with regards to their role in intestinal function or general health. Two of the *Prevotella* species identified were not only under-represented, but were completely absent from the colon cancer samples analyzed. *Prevotella* was a dominant genera reported in stool from children in a rural community in Burkina Faso but absent from a cohort of Italian children, and the study authors hypothesized that *Prevotella* helped maximize energy harvest from a plant-based diet [179]. Therefore, it is possible that the higher levels of *Prevotella* in the healthy cohort may reflect differences in the intake of fiber and other plant compounds compared to the individuals with colon cancer. At the genus level, Shen et al [180] found the *Bacteroides spp.* to be enriched in colonic tissue from healthy individuals when compared to adenoma tissue. Lachnospiracae and members of the genera *Dorea* and *Ruminococcus* were also previosly reported as dominant phylotypes driving differences between healthy and cancerous tissue samples [163]. The other OTUs that we identified such as the *Dialister spp.* and *Megamonas spp*. have not previously been reported in association with colon cancer; however, decreased populations of *Dialister invisus* have been reported in Crohn's disease [181].

There were fewer identifiable bacteria that were over-represented in the colon cancer population (Table 3.4). Most notably, we observed that the mucin-degrading bacteria, *Akkermansia muciniphila*, which represented a relatively large percentage of the total sequences, was present in a significantly greater proportion in the feces of colon cancer patients. This bacterium is a common member of the colonic microbiota and was recently shown to be reduced in irritable bowel syndrome and Crohn's Disease [182]; however a more recent report showed

increased *A. muciniphila* in ulcerative colitis-associated pouchitis [183]. Two types of mucins, MUC1and MUC5AC, are reportedely overexpressed in colon cancers [184], suggesting that our observed CRC-related increases in *A. muciniphila* populations may be due to increased substrate availability. *Citrobacter farmeri*, which can utilize citrate as a sole carbon source was also higher in samples from colon cancer patients, but represented a much smaller proportion of the total bacterial sequences. *Citrobacter farmeri* is among a group of gut bacteria that includes multiple pathogenic species like *Salmonella* and *Shigella,* and which has arylamine *N*-acetyltransferase activity that may be involved in activation of carcinogens and xenobiotic metabolism [185].

Age and BMI represent other factors that play a role in shaping the intestinal microbial communities. Several reports have demonstrated a correlation between the ratio of Bacteroidetes to Firmicutes and obesity [1]. We conducted linear regressions between the relative abundance of each of the taxa that significantly differed between CRC and healthy stools (see Tables 3.3 and 3.4) and BMI and saw no significant correlations (Table 3.5). In addition, aging has been associated with a decrease in protective commensal anaerobes, such as *Feacalibacterium prausnitzii,* and an increase in *E. coli* [186]. We did find a negative correlation between the age of participants and *Dorea formicagens* (R^2=0.354; p=0.041) and *Ruminococcus obeum* (R^2=0.434; p=0.020), both members of the Clostridium XIVa group, suggesting that differences between cohorts with regard to these two species may be a result of differences in the mean age of participants in each group rather than CRC disease status. To our knowledge, a decline in the population of Clostridium XIVa group members has not been previously associated with aging, but has been associated with dysbiosis related to intestinal inflammatory conditions such as Crohn's disease [187]. None of the other bacterial taxa identified were correlated with age (Table 3.6). Therefore, we conclude that the majority of taxa that significantly differed in stool samples

between healthy and CRC cohorts was a result of disease status and not of differences in age or BMI.

Short Chain Fatty Acid Analysis.

Short chain fatty acids (SCFA), particularly butyrate, are widely studied microbial metabolites reported to have anti-tumorigenic effects [188]. SCFA's are readily absorbed and utilized in host tissues so detection in stool is typically considered an indication of production in excess of that which can be utilized by the host [179]. We and others [64,163] have observed that species of butyrate producing bacteria, such as *Ruminococcus spp*. and *Pseudobutyrivibrio ruminis*, were lower in stool samples from CRC patients compared to healthy controls. Therefore, we quantified several short chain fatty acids from frozen stool samples. The three major SCFAs produced as microbial metabolites, acetate, propionate, and butyrate, were all detected as were valeric, isobutyric, isovaleric, caproic, and heptanoic acids. Among these, acetic and valeric acids were significantly higher in stool samples from CRC patients ($p<0.0001$ and $p=0.024$ respectively) while butyric acid was significantly higher in the feces of healthy individuals ($p<0.0001$; Figure 3.3). No differences in propionic acid were detected between the two groups. Butyrate is regarded as one of the most important nutrients for normal colonocytes, and alone or in combination with propionate it has been shown to reduce proliferation and induce apotosis in human colon carcinomas [189]. Although acetate is an important SCFA for maintaining colonic health and as a precurser molecule for endogenous cholesterol production, elevated levels of this metabolite have previously been associated with CRC in humans [190]. Acetate can be used to produce butyrate and proportional differences in these metabolites between CRC and healthy samples may reflect a depletion of colonic microbes that can carry out this reaction in CRC

samples or it may be a result of degradation of butyrate to acetate under low colonic pH associated with CRC. We also observed significantly higher relative concentrations of isobutyric ($p<0.0001$) and isovaleric acid ($p=0.002$) in samples from individuals with CRC (Figure 3.3). These two SCFA's result from bacterial metabolism of branched chain amino acids valine and leucine, which were also higher in CRC stool samples (Table 3.7), and may account for the significant increases observed in these two SCFAs in the CRC population.

Global stool metabolites.

Stool samples allow for evaluation of bacteria residing in the intestinal lumen, and therefore, stool small molecules are considered to result from co-metabolism or metabolic exchange between microbes and host cells [163]. Global metabolite profiling performed herein on lyophilized stool samples provided insights into the relationship between microbial populations and metabolites, and lend to the identification of novel CRC metabolic biomarkers. The supervised multivariate analysis technique, Orthogonal Projection to Latent Structures-Discriminant Analysis (OPLS-DA), which facilitates interpretation by separately modeling predictive and orthogonal (non-predictive) variables, was used to determine if non-targeted GC-MS profiles were predictive of disease state of the donor. The OPLS-DA demonstrated satisfactory modeling and predictive capabilities for this dataset ($R2Y=0.986$; $QY2=0.927$), revealing a distinct separation between stool metabolic features of the two groups (Figure 3.4), suggesting that presence or absence of CRC is an important factor driving the variability in stool metabolites.

Compared to healthy controls, stool metabolome analysis revealed 11 amino acids that showed a 41-80% increase in stool samples of individuals with CRC (Table 3.7). Reasons that

could account for this CRC-associated increase in amino acid concentrations may include, but not be limited to differences in protein consumption patterns, inflammation-induced reduction in nutrient absorption, and increased autophagy associated with tumor cells resulting in accumulation of free amino acid pools [191]. Microbial degradation of dietary proteins in the distal colon is a putrefactive process that results in the production of toxic amines, and may account for the increased free amino acids we observed in CRC stool samples. An increased concentration of all amino acids except glutamine was previously reported in stomach and colon tumor tissues compared to healthy tissue [192]. The authors hypothesized that tumor cells may exhibit increased glutaminase activity resulting in glutamine conversion to glutamate. Consistent with these findings, we also saw a large increase, approximately 76%, in glutamate without a corresponding increase in glutamine in stool samples from colon cancer patients. Another recent study using NMR to identify and detect metabolites from stool water extracts from healthy and CRC samples showed that the CRC samples had approximately 1.5-fold higher levels of cysteine, proline, and leucine [193]. The increased concentrations of proline, serine, and threonine that were observed in CRC samples could also be the result from degradation of intestinal mucins, which are primarily comprised of glycoproteins rich in these amino acids [194]. This is consistent with the enrichment of *Akkermansia muciniphila*, a mucin-degrading bacteria, observed in CRC stool samples; although we saw no strong correlations between the relative proportion of these bacteria and specific amino acid concentrations.

There were higher levels of glycerol as well as several unsaturated fatty acids detected in the stool samples of healthy individuals. Human cancer cells have a known transport system for the uptake of glycerol, suggesting stool glycerol may be lower in CRC because it is being taken up by the tumor cells. Alternatively, bacterial lipases present in healthy individuals may facilitate

the metabolism of dietary and endogenously produced triacylglycerols, resulting in the final degradation products of glycerol and free fatty acids. In addition to glycerol, fatty acids most closely matching metabolomic signatures for linoleic acid, and stereoisomers of oleic acid were also higher in controls (Table 3.7). Finally, ursodeoxycholic acid (UDCA), a secondary bile acid produced by intestinal bacteria was approximately 63% higher in healthy individuals compared to CRC. While several bile acids such as lithicolic acid and deoxycholic acid have been associated with tumorigenesis, UDCA has shown chemopreventive effects in preclinical and animal models of CRC [195].

Correlation analysis of the microbiome and metabolome data revealed strong associations between some members of the stool microbiota and candidate metabolites. *Bacteroides finegoldii,* two *Dialister spp.*, and *P. ruminis* were strongly correlated, and *Bacteroides intestinalis* and *Ruminococcus obeum* were moderately correlated with increased stool free fatty acids and glycerol (Figure 3.5). These same bacteria were inversely associated with a cholesterol derivative and one or more of the amino acids that were overrepresented in stool samples from CRC patients. The two *Ruminococcus spp.* also showed a strong positive correlation with the presence of UDCA, in concurrence with previous reports that *Ruminoccoccus* species exhibit 7a- and 7b-hydroxysteroid dehydrogenase activities to produce this metabolite [196]. Two of the bacterial genera overrepresented in CRC, *Phascolarctobacterium* and *Acidiminobacter* showed a strong positive association with the amino acids phenylalanine and glutamate, and were moderately correlated with increased serine and threonine (Figure 3.5). Glutamate can be utilized by these bacteria as a substrate, but their association with serine and threonine could also be indicative of involvement in mucin degradation or putrificative processes in the colon and warrant further study.

Extensive attempts to characterize CRC microbiota have led to new hypotheses as to how the gut microbiota influences CRC development. One hypothesis suggests that there are “driver bacteria” with pro-carcinogenic features that contribute to tumor development and “passenger bacteria” that may outcompete drivers to flourish in the tumor environment as the cancer progresses [197]. Available metabolites, those produced by bacteria and those that they utillize as substrates will largely drive these host-microbiome interactions. Integrating metabolome and microbiome datasets is a novel approach towards finding new directions to functionally characterize the microbiota in terms of their metabolic activity relative to cancer will greatly assist in our understanding of this complex host-microbe interaction.

Figures

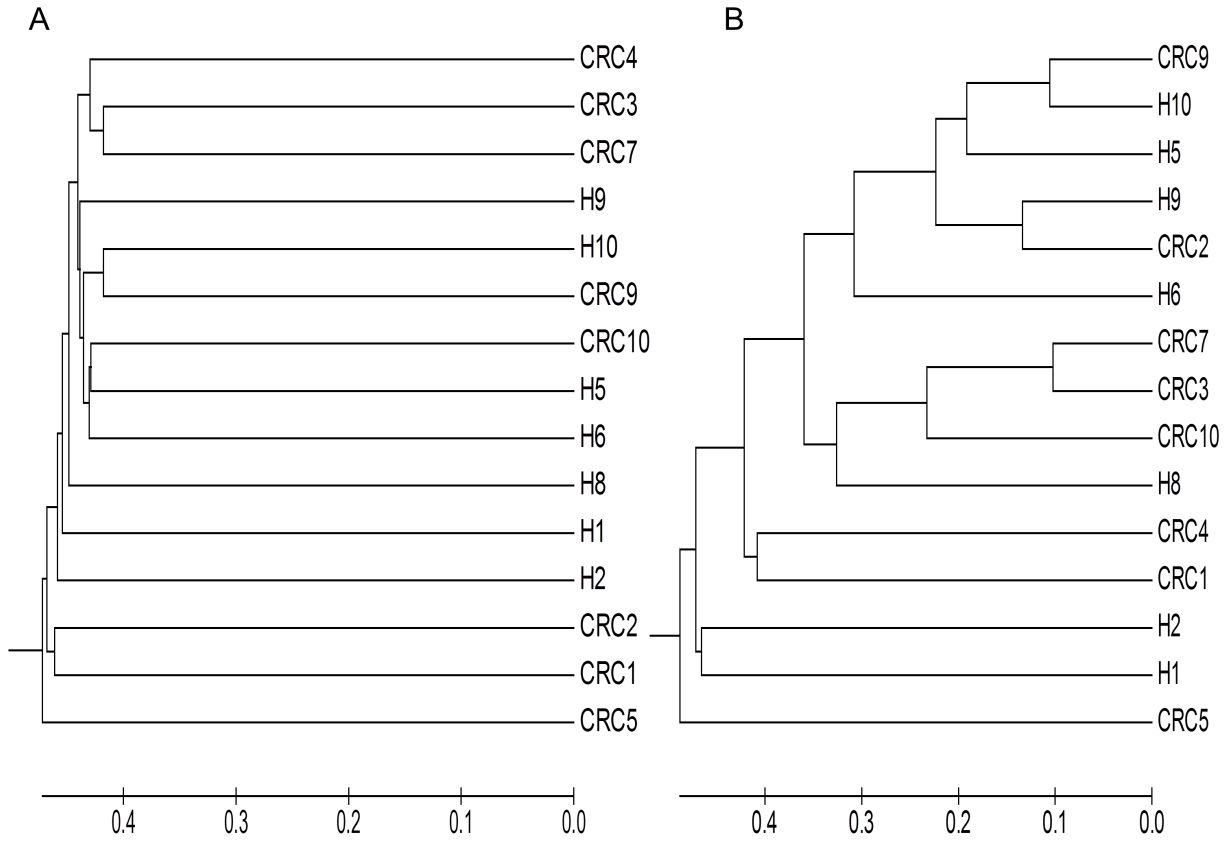


Figure 3.1. Using the 3% genetic distance, we observed no clustering of samples according to total stool microbial communities based on disease status of the sample donor using either the unweighted measure Jaccard similarity (A) or the weighted $Theta_{YC}$ distance (B).

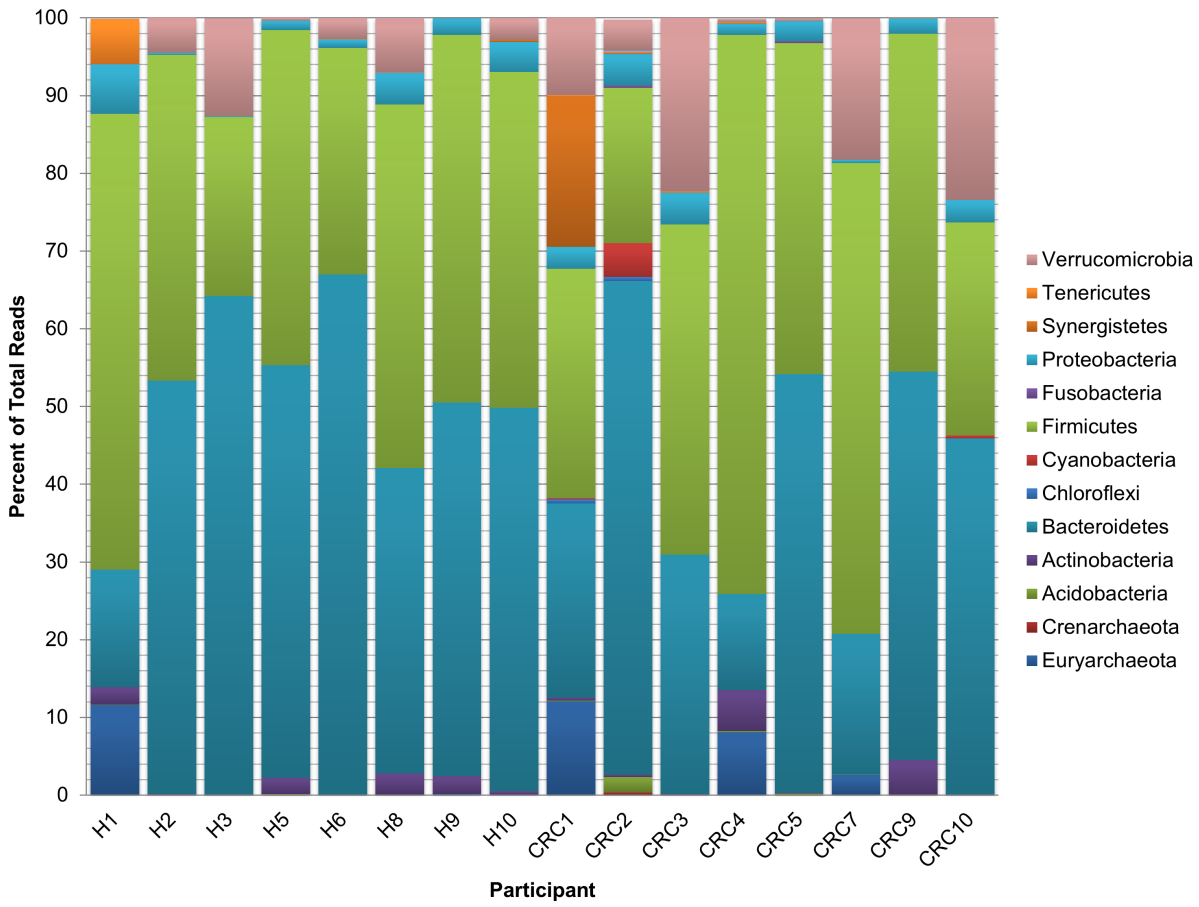


Figure 3.2. Phyla-level microbial classification of bacteria from individual stool samples. H sample numbers indicate samples from healthy adults while the C designation signifies samples from colon cancer patients.

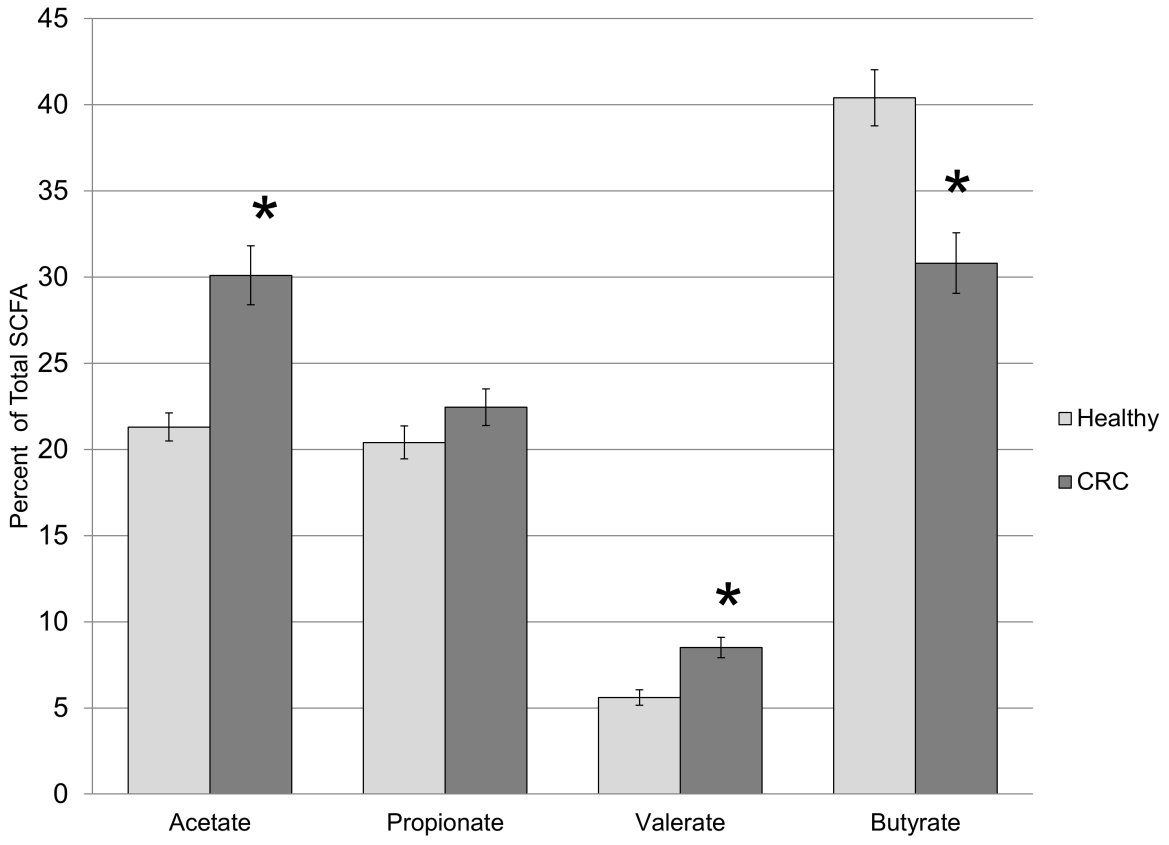


Figure 3.3. The relative proportion of bacterially-produced short chain fatty acids (SCFA) differed significantly between stool of healthy adults and individuals with CRC. Acetic acid, valeric acid, isobutyric acid, and isovaleric acid concentrations were proportionately higher while the anti-proliferative SCFA, butyric acid was significantly lower**.**

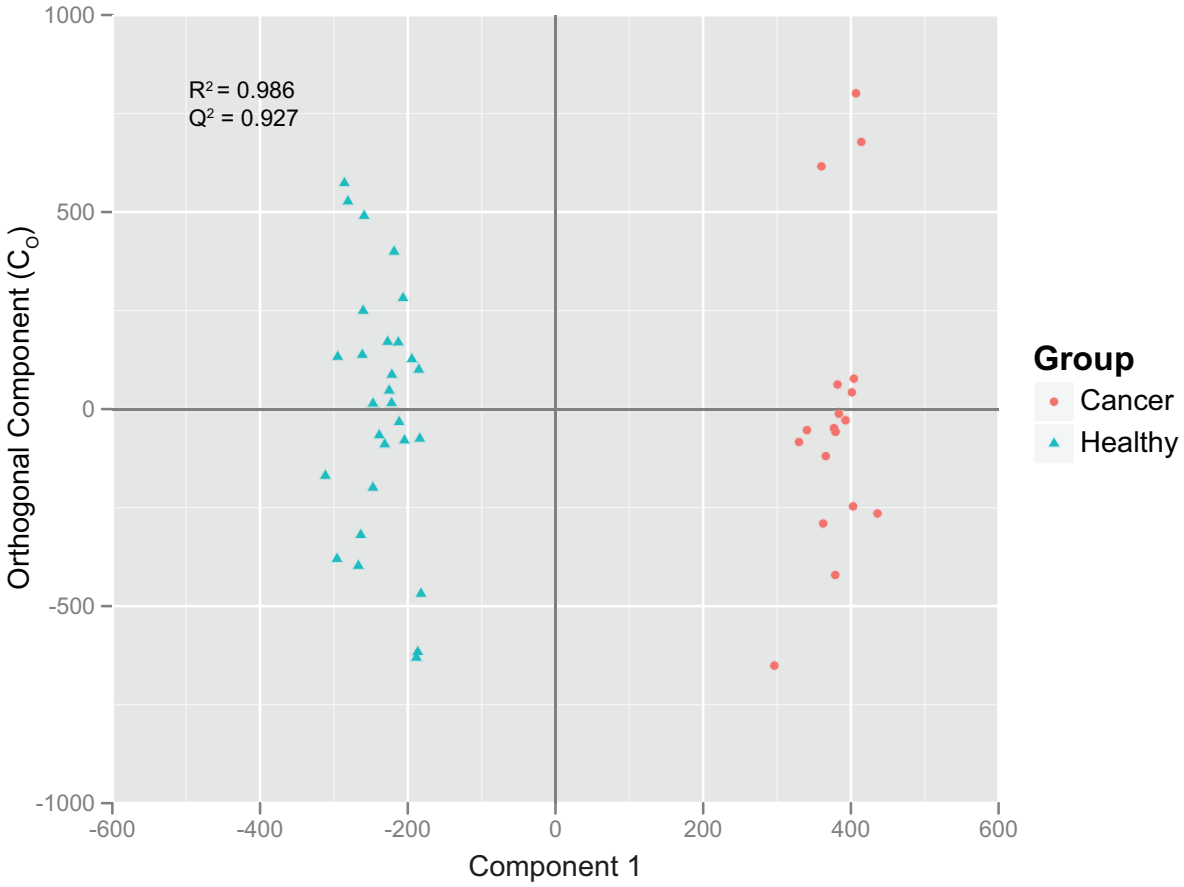


Figure 3.4. OPLS-DA scores plot generated from global GC-MS profiles differentiate stool metabolites from CRC patients and healthy adults.

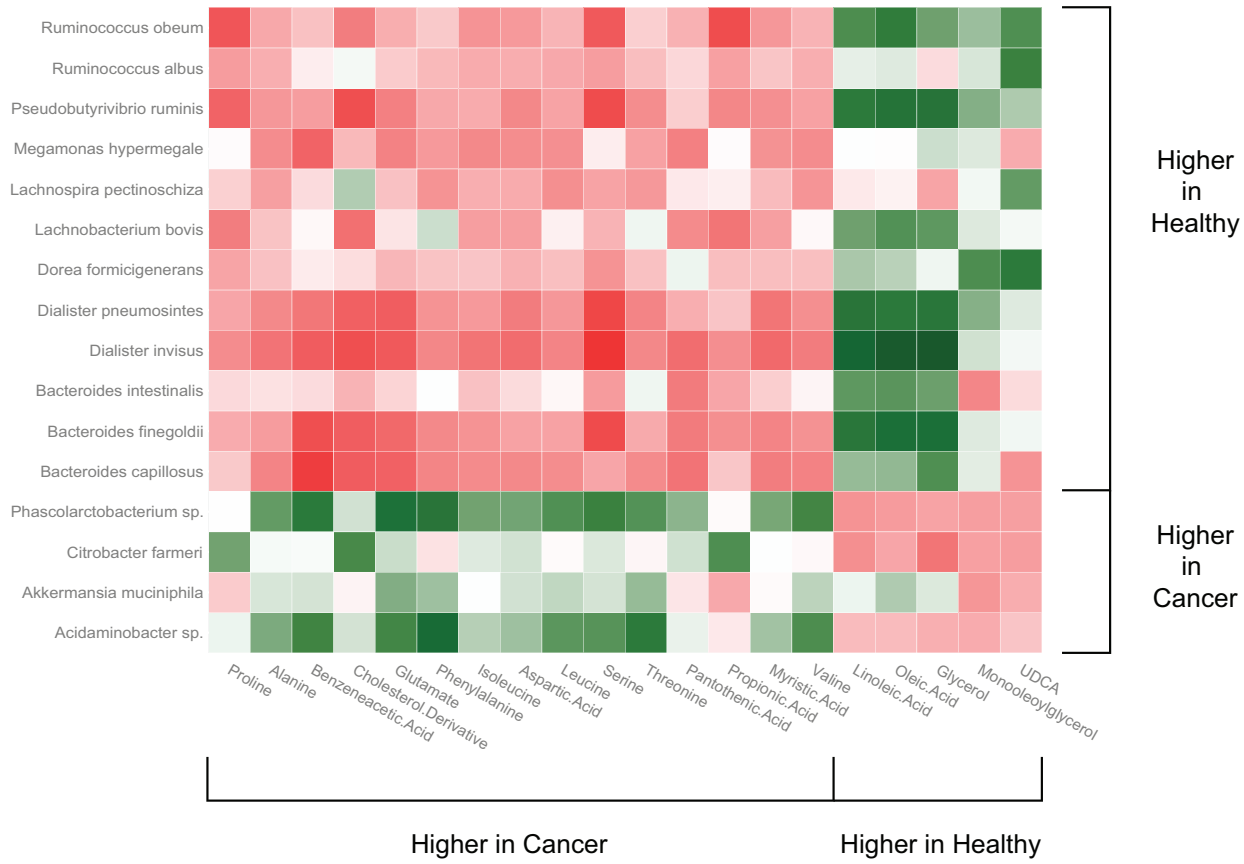


Figure 3.5. A heat map showing Pearson's correlations between groups of metabolites and bacterial genera/species that significantly differed between CRC patients and healthy adults. Green boxes indicate positive associations and red boxes indicate negative associations.

Tables

Table 3.1. Study participant characteristics.

Participant ID	Health Status	Sex	Age	BMI	Tumor Stage	Size (cm)	Tumor Location
CRC1[s]	Cancer	M	84	43.3	T2	5	Rectum
CRC2[s,t,g]	Cancer	M	67	39.1	T2	1	Ascending
CRC3[s,t,g]	Cancer	M	47	36.2	T1	0.01	Sigmoid
CRC4[s,t,g]	Cancer	M	68	28.8	T2	2.8	Rectum
CRC5[s,t,g]	Cancer	M	85	26.2	T3	5.5	Ascending
CRC6[t,g]	Cancer	F	51	34	T1	0.5	Sigmoid
CRC7[s,t,g]	Cancer	M	74	28.6	T3	4.5	Sigmoid
CRC8[t,g]	Cancer	F	55	21.6	Tis	4.5	Sigmoid
CRC9[s,t,g]	Cancer	M	30	24.3	T3	1.7	Rectum
CRC10[s,t,g]	Cancer	M	76	26.2	T3	2.5	Ascending
H1[s,t]	Healthy	M	39	24.7			
H2[s,t,g]	Healthy	F	36	22.8			
H3[t,g]	Healthy	F	54	23.8			
H4[t,g]	Healthy	F	57	23			
H5[s,t,g]	Healthy	F	26	35.7			
H6[s,t,g]	Healthy	F	24	24.9			
H7[t,g]	Healthy	F	34	25.2			
H8[s,t,g]	Healthy	M	67	30.1			
H9[s,t,g]	Healthy	M	34	21.9			
H10[s,t,g]	Healthy	F	25	20			
H11[t,g]	Healthy	F	52	26.4			

Sample included in [s]454 pyrosequencing analysis; [t]targeted analysis of bacterial SCFA's, and [g]global metabolite profiling by GC-MS. Tis: Carcinoma in situ: intraepithelial or invasion of lamina propria; T1: Tumor invades submucosa; T2: Tumor invades muscularis propria; T3:Tumor invades through muscularis propria into the subserosa or into nonperitonealized pericolic or perirectal tissue.

Table 3.2. Bacterial species that were significantly more abundant in the stool of healthy individuals compared to CRC patients.

Bacterial Species	Avg. Healthy (%)	Avg. CRC (%)	Fold Change	p value
Bacteroides finegoldii	0.74	0.29	2.5	0.0032
Bacteroides intestinalis	0.53	0.19	2.9	0.0063
Prevotella copri	4.09	0	40	0.0000
Prevotella oris	1.64	0	16	0.0001
Ruminococcus obeum	0.62	0.34	1.8	0.0009
Dorea formicigenerans	0.24	0.08	2.9	0.0001
Lachnobacterium bovis	1.20	0.62	1.9	0.0002
Lachnospira pectinoschiza	0.54	0.21	2.6	0.0005
Pseudobutyrivibrio ruminis	0.39	0.12	3.2	0.0000
Bacteroides capillosus	0.23	0.10	2.2	0.0057
Ruminococcus albus	0.36	0.03	10.3	0.0008
Dialister invisus	3.45	0.07	48.7	0.0000
Dialister pneumosintes	0.48	0.01	52.6	0.0000
Megamonas hypermegale	0.24	<0.01	44.5	0.0001

Table 3.3. Comparison of observed and estimated OTU richness and diversity and evenness indices between microbial communities from stool of CRC patients and healthy adults.

					Shannon			Simpson		
Group	nseqs	coverage	S_{obs}	S_{chao}	H'	E_H	S_H	1-D	E_D	S_D
Healthy	1250	0.839	309	838	4.484	0.782	89	0.970	0.109	34
Healthy	1250	0.854	250	805	3.669	0.664	39	0.915	0.047	12
Healthy	1250	0.892	206	541	3.316	0.622	28	0.871	0.038	8
Healthy	1250	0.839	290	864	3.961	0.699	53	0.913	0.040	11
Healthy	1250	0.858	257	729	3.660	0.660	39	0.905	0.041	10
Healthy	1250	0.793	364	1243	4.663	0.791	106	0.973	0.103	38
Healthy	1250	0.817	325	1012	4.456	0.770	86	0.963	0.084	27
Cancer	1250	0.842	287	960	4.209	0.744	67	0.955	0.077	22
Cancer	1250	0.906	184	460	3.353	0.643	29	0.900	0.054	10
Cancer	1250	0.834	285	867	4.015	0.710	55	0.943	0.061	18
Cancer	1250	0.857	255	786	3.787	0.683	44	0.926	0.053	14
Cancer	1250	0.842	290	919	4.303	0.759	74	0.961	0.089	26
Cancer	1250	0.864	229	1127	3.582	0.659	36	0.925	0.058	13
Cancer	1250	0.861	263	670	3.822	0.686	46	0.919	0.047	12
Cancer	1250	0.867	232	803	3.674	0.675	39	0.926	0.058	14
TTEST(H:C)		0.24	0.18	0.73	0.41	0.54	0.27	0.90	0.78	0.44

Table 3.4. Bacterial species significantly over-represented in CRC stool samples.

Bacterial Species	Avg. Healthy (%)	Avg. CRC (%)	Fold Change	p value
Acidaminobacter unclassified	0.05	0.39	7.7	0.0045
Phascolarctobacterium unclassified	3.31	11.0	3.2	0.0000
Citrobacter farmeri	0.08	0.37	4.6	0.0050
Akkermansia muciniphila	3.54	12.8	3.6	0.0032

Table 3.5. Candidate stool metabolites identified from GC-MS chromatograms that differ between CRC and healthy individuals.

Candidate	**Chemical Class**	**% change in CRC**	***p value***
Alanine	Amino Acid	74.0	<0.001
Glutamate	Amino Acid	76.1	<0.0001
Glycine	Amino Acid	72.3	<0.01
Aspartic acid	Amino Acid	82.2	<0.0001
Leucine	Amino Acid	61.0	<0.005
Lysine	Amino Acid	59.2	<0.05
Proline	Amino Acid	85.0	<0.001
Serine	Amino Acid	41.6	<0.005
Threonine	Amino Acid	79.7	<0.001
Valine	Amino Acid	73.0	<0.001
Phenylalanine	Amino Acid	77.3	<0.001
Benzeneacetic Acid	Carboxylic Acid	42.5	<0.005
Propionic acid	Short Chain Fatty Acid	74.2	<0.001
Myristic Acid	Saturated Fatty Acid	61.3	<0.001
Pantothenic acid	Vitamin B5	46.5	<0.01
Cholesterol derivative	Steroid	45.2	<0.005
Oleic acid*	unsaturated fatty acid	-74.6	<0.05
Linoleic acid*	unsaturated fatty acid	-67.3	<0.005
Elaidic acid*	unsaturated fatty acid	-45.5	<0.005
Glycerol	Polyol	-53.3	<0.005
Monooleoylglycerol	Polyol derivative	-55.4	<0.01
Ursodeoxycholic acid	Bile acid	-63.1%	<0.005

*Fatty acid identifications were conducted at a level that does not distinguish bond placement.

Table 3.6. Linear regressions of selected bacterial taxa with participant BMI.

Bacterial taxa	R^2 value	P value
Bacteroides finegoldii	0.129	0.251
Bacteroides intestinalis	0.105	0.303
Ruminococcus obeum	0.038	0.543
Dorea formicigenerans	0.003	0.868
Lachnobacterium bovis	0.071	0.403
Lachnospira pectinoschiza	0.113	0.285
Pseudobutyrivibrio ruminis	0.013	0.724
Bacteroides capillosus	0.165	0.190
Ruminococcus albus	0.161	0.196
Dialister invisus	0.065	0.424
Dialister pneumosintes	0.001	0.905
Megamonas hypermegale	0.197	0.149
Acidaminobacter unclassified	0.016	0.699
Phascolarctobacterium unclassified	0.056	0.457
Citrobacter farmeri	0.218	0.126
Akkermansia muciniphila	0.064	0.428

Table 3.7. Linear regressions of selected bacterial taxa with participant age.

Bacterial taxa	R^2 value	P value
Bacteroides finegoldii	0.181	0.167
Bacteroides intestinalis	0.001	0.935
Ruminococcus obeum	0.434	0.02
Dorea formicigenerans	0.354	0.041
Lachnobacterium bovis	0.07	0.407
Lachnospira pectinoschiza	0.202	0.143
Pseudobutyrivibrio ruminis	0.209	0.135
Bacteroides capillosus	0.065	0.423
Ruminococcus albus	0.307	0.061
Dialister invisus	0.113	0.286
Dialister pneumosintes	0.136	0.238
Megamonas hypermegale	0.052	0.457
Acidaminobacter unclassified	0.115	0.218
Phascolarctobacterium unclassified	0.181	0.168
Citrobacter farmeri	0.188	0.16
Akkermansia muciniphila	0.018	0.678

CHAPTER 4: PILOT DIETARY INTERVENTION WITH HEAT-STABILIZED RICE BRAN MODULATES STOOL MICROBIOTA AND METABOLITES IN HEALTHY ADULTS[5,6]

Summary

Heat-stabilized rice bran (SRB) has been shown to regulate blood lipids and glucose, modulate gut mucosal immunity and inhibit colorectal cancer in animal and human studies. However, SRB's effects on gut microbial composition and metabolism and the resulting implications for health remain largely unknown. A pilot, randomized-controlled trial was developed to investigate the effects of eating 30 g/day SRB on the stool microbiome and metabolome. Seven healthy participants consumed a study meal and snack daily for 28 days. The microbiome and metabolome were characterized using 454 pyrosequencing and gas

[5] A modified version of this chapter is published as Sheflin, A.M., Borresen, E.C., Wdowik, M.J., Rao, S., Brown, R.J., Heuberger, A.L., Broeckling, C.D., Weir, T.L. and Ryan, E.P., 2015. Pilot dietary intervention with heat-stabilized rice bran modulates stool microbiota and metabolites in healthy adults. Nutrients, 7(2), pp.1282-1300. The final publication and supporting material is available from MDPI at http://dx.doi.org/10.3390/nu7021282

[6] The second aim of this work was to describe changes in stool metabolites and microbiota in healthy adults after 2 and 4 weeks of supplementation with SRB. These results were interpreted for potential effects on intestinal health based upon the existing literature and results from Chapter 2.

This chapter includes the complete published manuscript for this research titled *Pilot dietary intervention with heat-stabilized rice bran modulates stool microbiota and metabolites in healthy adults* (Sheflin, A.M., Borresen, E.C., Wdowik, M.J., Rao, S., Brown, R.J., Heuberger, A.L., Broeckling, C.D., Weir, T.L. and Ryan, E.P., *Nutrients*, 2015). My contributions to this publication included the bulk of the analysis, statistics, and interpretation for all stool microbiota data and all metabolite data, except for SCFA. I wrote the bulk of the manuscript and created all the figures and tables except for Figure 1 and Tables 1 and 2.

Table and figure numbers have been modified to reflect that they are specific to this chapter, e.g. Figure 1 is now Figure 4.1. In addition, figures were renumbered to incorporate supplemental material into the flow of the chapter. This article was published under open access and is reproduced under the creative commons license. Only minimal modifications were made to meet formatting requirements. No other modifications were made.

This work is licensed under a Creative Commons Attribution 4.0 International License. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited. To view a copy of this license, visit https://creativecommons.org/licenses/by/4.0/

chromatography-mass spectrometry (GC-MS) at baseline, two and four weeks post-intervention. Increases in eight operational taxonomic units (OTUs), including three from *Bifidobacterium* and *Ruminococcus* genera, were observed after two and four weeks of SRB consumption ($p < 0.01$). Branched chain fatty acids, secondary bile acids and eleven other putative microbial metabolites were significantly elevated in the SRB group after four weeks. The largest metabolite change was a rice bran component, indole-2-carboxylic acid, which showed a mean 12% increase with SRB consumption. These data support the feasibility of dietary SRB intervention in adults and support that SRB consumption can affect gut microbial metabolism. These findings warrant future investigations of larger cohorts evaluating SRB's effects on intestinal health.

Introduction

Heat-stabilized rice bran (SRB) is a nutrient-dense and phytochemical-rich food ingredient that is not widely consumed, but is gaining attention for its potential to help prevent multiple chronic diseases, including cardiovascular disease [198], type 2 diabetes [199,200], metabolic syndrome [198,201,202] and cancer [6,203,204]. Heat stabilization increases the shelf life of rice bran through inactivation of rancidity-inducing lipases and lipoxygenases, while retaining bioactivity [41,42]. The bioactive components of rice bran include, but are not limited to, γ-oryzanol, tocopherols, tocotrienols, phenolics (e.g., ferulic acid, caffeic acid), phytosterols (e.g., beta-sitosterol, cycloartenol) and specific free amino acids [6]. Whole grain brown rice reportedly changes the composition of gut bacterial phyla [26], increases anti-inflammatory SCFA levels in an *in vitro* canine microbiome model [36] and is a source of prebiotics [205]. Unidentified non-starch components of whole grains, including brown rice, were found to elicit changes in bacterial diversity, the *Firmicutes/Bacteroides* ratio and the bacterial abundance of

the *Blautia* genus in the human gut microbiome [26]. In contrast to whole grain brown rice, SRB delivers a greater concentration of nutrients and phytochemicals to the gut and, as such, may differentially modulate stool microbiota and metabolites.

The structure and composition of the stool microbiome has shown profound associations with both human health and disease [69]. Emerging evidence supports the role of diet in modulating the structure of the gut microbiota [26,206,207]. Host diet, in turn, shapes not only the gut microbial composition, but also its metabolism [207]. Diet modifications have demonstrated effects on energy harvest, macronutrient metabolism and cancer risk, largely due to changes in microbially-produced metabolites that may promote or inhibit gastrointestinal health outcomes [67,208]. In particular, microbial metabolites have been found to exert pro- or anti-inflammatory activity on intestinal tissues and influence barrier function [209], host immune response [210,211], tumorigenesis [133,212] and tumor proliferation [213]. These metabolic perturbations result from changes in substrate availability, as well as diversity amongst microorganisms to ferment and biotransform specific dietary components. Dietary modulation of the microbiome and associated metabolism may prove a viable strategy for disease prevention and optimization of health.

The objective of this pilot dietary SRB intervention was to confirm the acceptability and feasibility of SRB supplementation in people and to assess the potential effects of the diet on the composition of the stool microbiome and metabolome. We hypothesized that SRB consumption would promote microbial changes and alter stool metabolite profiles. These alterations in stool microorganisms and metabolites may explain the benefits of SRB for intestinal health and account for the reported SRB bioactivities in preventing chronic disease. Findings from this pilot

study provide compelling support and the rationale for larger cohort investigations of dietary SRB supplementation.

Materials and Methods

Pilot Trial Design and Participation

A four-week, pilot, randomized-controlled, single-blinded dietary SRB intervention study was completed in seven healthy adults with no history of cancer at Colorado State University (CSU) and is part of a community-based collaboration with the University of Colorado Health-North (UCH) in Fort Collins, CO, USA. Inclusion criteria for participants included no history of food allergies or major dietary restrictions, not currently taking cholesterol-lowering medications or non-steroidal anti-inflammatory drugs (NSAIDs), not currently pregnant or lactating, not a current smoker, no antibiotic use or probiotic use within the last month and no history of gallstones. The CSU Research Integrity and Compliance Review Office and the UCH-North Institutional Review Board approved this study protocol (CSU protocol #: 09-1520H, 02/18/2010, and UCH-North IRB #: 10-1038, 07/28/2010). Written informed consent was obtained from all participants prior to enrollment.

Participants received study meals and snacks that either included SRB (30 g/day) or that did not include SRB (control). Participants were instructed to consume one study-provided meal and snack each day and were not required to alter the remainder of their other daily food intake. To remain blinded to the intervention, the study-provided meals and snacks were labeled “Group A” or “Group C”. Participants self-recorded the amount of each study meal and snack consumed daily for compliance assessment. Participants also completed a 3-day food log (2 weekdays, one weekend day) each week to more accurately measure total dietary intakes. Food logs were

entered and analyzed using Nutritionist Pro™ diet analysis software (Axxya Systems, Redmond, WA, USA), and each diet log analysis included average daily caloric intake, macronutrient, amino acid, vitamin and mineral profiles. Participants self-collected stool samples in coded specimen containers within 24 h of their scheduled study visit and provided the sample to the study coordinator at three required time points: baseline, 2 weeks and 4 weeks. Stool specimens were refrigerated by participants prior to arrival; DNA and SCFA aliquots were extracted within 24 h; and the remainder of the stool sample was stored at −20 °C until lyophilization and global metabolite extraction. All participants completed the trial without any reported adverse effects.

Heat Stabilization of Rice Bran

Rice bran was provided by the US Department of Agriculture-Agricultural Research Service (USDA-ARS) Dale Bumpers Rice Research Unit (Anna McClung) and was derived from a single source (*Oryza sativa* L. ssp. *japonica* var. Neptune). The rice bran was heat-stabilized by heating at 100 °C for three min to inactivate the lipase/lipoxygenase enzymes and prevent the bran from becoming rancid.

Composition of SRB and Control Intervention Meals and Snacks

Seven meals (e.g., casseroles, soups) and six snacks (e.g., smoothies, granola, crackers) were developed by a registered dietitian and certified chef for both the absence and inclusion of SRB and covered a wide range of taste preferences. The placebo-control meals and snacks were similarly matched in their macronutrient content to the intervention and did not contain any SRB or brown rice. Similar palatability and appearance of both control and SRB meals and snacks were confirmed with a community taste test, including people with and without a history of

cancer. These recipe and taste test trials were conducted in accordance with IRB-approved protocols (data not shown). Recipes for the dietary intervention meals and snacks were analyzed using NutritionistPro™ diet analysis software (Axxya Systems, Stafford, TX, USA). Each intervention meal and snack contained 15 g of SRB to achieve a total daily intake of 30 g.

DNA Extraction, Amplification and Sequencing

Stool samples were subsampled three times with sterile cotton swabs. MoBio Powersoil DNA extraction kits (MoBio, Carlsbad, CA, USA) were utilized according to the manufacturer's instructions to extract DNA. Sample DNA was stored at −20 °C prior to amplification steps. Sequencing libraries were prepared as described in [65]. Sequencing was conducted under contract by the University of South Carolina's Engencore Sequencing Facility using a 454 Life Sciences GS FLX System with titanium chemistry.

Analysis of Microbiota

All bacterial sequence read editing and processing was performed with mothur Ver. 1.28 [214] using the default settings, unless otherwise noted. The mothur software package is an open source bioinformatics tool used to analyze 16S rRNA gene sequences. Briefly, sequence reads were: (i) trimmed (options used with the trim command in mothur were as follows: bdiff = 1, pdiff = 2, qaverage = 30, minlength = 200, maxambig = 0, maxhomop = 8, flip = T); (ii) aligned to the bacterial-subset SILVA alignment [215]; (iii) filtered to remove vertical gaps; (iv) screened for potential chimeras using the uchime method; (v) classified using the Ribosomal Database Project's naïve Bayesian classifier (RDP-NBC) training set for mothur [216]; all sequences identified as chloroplast were removed; (vi) sequences were screened (optimize =

minlength-end, criteria = 95) and filtered (vertical = T, trump = .), so that all sequences covered the same genetic space; and (vii) all sequences were pre-clustered (with up to two base-pair mismatches using the option diff = 2) to remove potential pyrosequencing noise and clustered into OTUs based on a 3% distance cutoff using the average-neighbor algorithm [217]. To remove the effect of sample size on community composition metrics, sub-samples of 450 reads were randomly selected from each stool sample. Sub-sampled community metrics were used to calculate alpha diversity community descriptors, including observed species richness (Sobs), Shannon's diversity (H') and evenness (E_H) and Simpson's diversity (S_D) using the mothur implementation of these calculators. All sequence data are publicly available through the Sequence Read Archive (SRA) under study Accession Number PRJEB8075, which is available at the following link: http://www.ebi.ac.uk/ena/data/view/ERP002217.

Metabolite Extraction and Detection by Gas Chromatography-Mass Spectrometry

Lyophilized stool metabolites were extracted using 80:20 MeOH:H_2O and detected using GC-MS, as described previously [65]. For each sample, a matrix of molecular features defined by retention time and mass (*m/z*) was generated using XCMS software [218]. Features were normalized to total ion current, and the relative quantity of each molecular feature was determined by the mean area of the chromatographic peak among replicate injections ($n = 2$). Metabolites were identified by matching mass spectra to the National Institute for Technology Standards (www.nist.gov) and Golm (http://gmd.mpimp-golm.mpg.de/) metabolite databases [33] after deconvolution using AMDIS software [219].

Short Chain Fatty Acid Determination

Frozen stool samples were extracted for short chain and branched chain fatty acids (SCFA and BCFA, respectively) using acidified water (pH 2.5), as described previously [65]. Peak areas were normalized to the total signal and represented as a percentage of total SCFA. Commercial standards of acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, caproic acid and heptanoic acid (Sigma, St. Louis, MO, USA) were used to confirm compound identities.

Statistical Analysis and Data Visualization

The data on caloric intake, macronutrients and SCFA were checked for assumptions of normality. Due to the small sample size and non-normality, the SCFAs data were converted into ranks prior to performing a linear regression analysis. The "diet" groups and "time points" along with their interaction terms were used as factors to evaluate their impact on SCFA outcomes. Medians were used to describe the data. The analysis took into account the repeated measures on the same individual over time. A *p*-value of 0.05 was considered for determining statistical significance.

Microbiota Analyses

The mothur implementation of the analysis of molecular variance (AMOVA) test, using a *p*-value threshold of 0.01, was applied to determine variation in community samples. AMOVA is a non-parametric version of the traditional analysis of variance (ANOVA) test that is widely used when testing genetic diversity. PCoA loadings were generated in mothur and visualized using R software (v3.0.1) [220]. The METASTATS [221] function within mothur was used to detect

differentially abundant bacterial taxa in stool from SRB-consuming participants *versus* controls at a corrected *p*-value (expressed as the *q*-value and calculated based on a published algorithm [222]) threshold of 0.05.

Metabolome Analyses

Stool metabolite features were identified via GC-MS. Differences in metabolite features between samples from baseline to 4 weeks in the SRB and control groups were determined using the Student's *t*-test with a significance cutoff of <0.01. To minimize baseline inter-individual differences, metabolite data were first converted to the percent change from baseline that was calculated using the normalized area under the curve (AUC) as follows:

$$(\text{4 week AUC} - \text{Baseline AUC})/(\text{Baseline AUC}) \times 100 = \% \text{ total}$$

Negative values represent a decrease from baseline in a particular metabolite. In order to focus on metabolite changes due primarily to SRB intake rather than other food components that were common to the intervention meals and snacks, the mean change for each metabolite in the control group was subtracted from the mean change in the SRB group.

Individual SCFA concentrations were normalized as a percent of total measured SCFAs, and a weighted mean was calculated for each quantified compound. Statistical differences ($p < 0.05$) between stool samples from SRB-consuming participants and control participants were determined using a mixed model ANOVA representing a random effect and SRB intervention as a fixed effect (XLSTAT 2011.1, Addingsoft Corp, Paris, France).

Results

Completion of this pilot study demonstrated the feasibility of the placebo control, single-blinded dietary SRB intervention in healthy adults and established a standardized collection of stool samples for microbiome and metabolome assessment. All seven participants completed this pilot study between August 2010 and March 2011. Three participants were allocated to the control diet, and four participants were allocated to the SRB diet. Participant baseline characteristics are shown in Table 4.1.

Increased SRB Effects on Caloric and Macronutrient Intakes

Participants randomized to the SRB group consumed 30 g of SRB daily for the 28-day duration of the study, which compositionally included 6.26 g fat, 4.01 g protein, 14.91 g carbohydrate and a variety of vitamins and minerals (Figure 4.1) [223]. Even though all participants were free to consume brown rice or SRB outside of the intervention, the three-day food logs revealed that none of the participants from the control or SRB group were consuming whole grain brown rice during the study. The three-day food log analysis collected from participants each week revealed no significant change in caloric intake from Week 2 to Week 4 for either the control ($p = 0.455$) or SRB groups ($p = 0.620$). There was no significance between groups at the two-week or four-week time points ($p = 0.966$ and $p = 0.394$, respectively). Table 4.2 shows the caloric intakes for both groups. The median caloric intake for the SRB group was 1941 kcal at Week 2 and 1791 at Week 4. The control group had a median caloric intake of 2186 kcal at Week 2 and 2099 at Week 4. The control group had a significant decrease in protein intake at Week 4 compared to Week 2 ($p = 0.001$), and the SRB group had a significant decrease in

protein intake at Week 4 compared to Week 2 ($p < 0.0001$). Carbohydrate intakes were not significantly different in the control group compared to the SRB group at Week 4 ($p = 0.7$). A similar pattern was shown for total fat intake at Week 4 ($p = 0.99$). The control group had a significant increase ($p = 0.021$) of fat intake at Week 4 compared to Week 2. The SRB group had significantly higher fiber intakes at Week 2 ($p < 0.0001$) when compared to the Week 2 control group, as well as at Week 4 compared to the control ($p = 0.04$).

Microbiome Changes with Consumption of SRB

On average, the coverage of the stool microbial community was 89% (Table 4.5), and after subsampling, 2160 operational taxonomic units (OTUs) were detected in total. Stool bacterial richness, evenness and diversity remained constant during the SRB intervention with these healthy participants (Table 4.3). The composition of the stool microbial communities at the phylum levels showed a high level of individual variation at both baseline and during the dietary intervention (Figure 4.2). Comparing bacterial composition at two and four weeks to the baseline at the phyla level revealed no significant changes in either SRB or control participants. After two weeks, eight OTUs belonging to the genera *Methanobrevibacter*, *Paraprevotella*, *Ruminococcus*, *Dialister*, *Anaerostipes* and *Barnesiella* showed significantly increased abundance (Table 4.4), and no OTUs showed reduced abundance with SRB. Additionally, increases in OTUs from the genera *Bifidobacterium* and *Clostridium* were noted at four weeks compared to the baseline. No significant changes at any taxonomic level were detected in stool bacterial composition for control participants.

Metabolome Changes with Increased SRB

SCFAs, particularly acetate, propionate and butyrate, are primary products of carbohydrate fermentation [224]. Given that SRB's macronutrient composition is 50% carbohydrate [41], SCFAs were quantified to assess changes driven by the SRB intervention. No significant increases in acetic, propionic, valeric, caproic and heptanoic acids were observed at two weeks or four weeks with the SRB intervention when compared to the baseline (Figure 4.3); however, butyric acid significantly decreased at Week 4 compared to the baseline and Week 2 (p = 0.025 and p = 0.0007, respectively). Furthermore, significant increases ($p < 0.05$) in isovaleric and isobutyric acid, both BCFAs, were observed at the two- and four-week rice bran intervention time points (Figure 4.3 and Table 4.5).

Non-targeted metabolic profiling using GC-MS has been previously shown to identify stool metabolites that vary due to dietary modifications and demonstrated co-metabolic interactions between host and gut microbiota [225]. In order to focus on metabolite changes due primarily to SRB intake and not other foods included in intervention meals and snacks, the average change for each metabolite in the control group was subtracted from the changes in the SRB group. Despite this conservative approach, significant increases in abundance (as the mean peak area) for 28 stool metabolites (in addition to the increases in BCFA detailed above) were revealed at four weeks in the SRB group compared to the baseline. Furthermore, significant decreases were observed for eight additional stool metabolites. These metabolites function in pathways concerning synthesis, digestion and/or degradation of: amino acids, cholesterol and bile acids, phytochemicals and phenolics, lipids, vitamins and minerals and carbohydrates (Table 4.6). The metabolite with the largest increase was indole-2-carboxylic acid, a known phenolic

component of whole grain rice [226], and this was increased approximately 12% at four weeks compared to the baseline (Table 4.6).

Significantly changed metabolites related to amine metabolic pathways were generally associated with purine and pyrimidine metabolism (Table 4.6). Decreases in stool leucine and glycine levels were observed in SRB participants, while BCFA metabolites of leucine, isoleucine and valine (Branched Chain Amino Acids) increased, as previously noted. Cholesterol and bile acid metabolites also increased with SRB (Table 4.6). Several beneficial phytochemicals increased with SRB, including two phytosterols known to be present in rice bran (Table 4.6) and the phenolics hydrocinnamic acid, benzoic acid and phenylacetic acid. Additionally, inositol phosphate was increased with SRB and is a potential product of phytic acid degradation, a known component of SRB. Metabolites in the lipid digestion and synthesis pathways also changed with SRB, including significant increases from the baseline for five saturated fatty acids (SFA) ranging from 1.0%–7.0 % (Table 4.6). Palmitic acid is a prominent component of SRB [227] and was found to be increased in stool following SRB consumption in this study (Table 4.6). Minor, but significant, increases were also observed for oleic acid and glycerol. Three fatty acids were seen to significantly decrease in stool after SRB intake: sebacic acid, 2-hexendioic acid and pentadecanoic acid.

Discussion

The primary goal of this pilot dietary intervention was to confirm the feasibility of SRB consumption at 30 g/day in healthy adults and to determine if this amount was sufficient to induce detectable differences in stool microbiota and metabolites. Significant increases in eight OTUs were identified from human stool microbiota analysis at four weeks. There were nine stool

metabolites increased and confirmed as SRB components and an additional eleven metabolite products of microbial metabolism that were elevated in stool at four weeks. The lack of adverse events and large-scale microbial community disruptions following the SRB intervention provide evidence for the feasibility of SRB consumption at 30 g/day for adults. Another goal of this pilot study was to set the stage with targeted microbial and metabolite endpoints that may influence intestinal health and colorectal cancer prevention outcomes. Results from this pilot study provided valuable insight into potentially important health-related changes that can be confirmed in future studies with larger sample sizes.

While there were no prior studies reporting the effect of SRB consumption on human microbiota, a human feeding study of 21 g/day of *Aspergillus*-fermented rice bran consumed for two weeks revealed no changes to the stool microbiota or SCFA profiles [228]. A dietary intervention using whole grain brown rice (60 g/day) for four weeks in human participants also produced no significant changes at the species level in participants' gut microbiota or SCFA profiles [26]. In contrast, the present intervention detected changes in 6–8 OTUs at both two weeks and four weeks and significantly decreased butyric acid at four weeks. The differing outcomes may be explained by the amount (30 g/day) and rice fraction type (*i.e.*, SRB *vs.* whole grain), as compared to previous approaches. Confirming changes to bacterial species abundance and levels of branched and short chain fatty acids with SRB in a larger dietary intervention will be valuable.

BCFA levels increased in SRB participants' stools and corroborate the 25% higher BCFA produced from rice compared to wheat, rye, corn and oats when subjected to *in vitro* digestion and fermentation using human stool samples [229]. The higher content of branched chain amino acids, valine and leucine, relative to other amino acids in SRB (Figure 4.1) may account for the

significantly increased BCFA production (Figure 4.3 and Table 4.5) by gut commensals. In general, increased BCFAs are associated with diets high in animal fat and are consistent with protein degradation in the colon [230]. However, there is no direct evidence that BCFAs are causal of negative health outcomes. In fact, they may play a role in preventing human intestinal disease processes, as BCFAs were found in lower amounts in patients with irritable bowel syndrome (IBS) when compared to healthy controls [231]. Barrier function was investigated with human Caco-2 cells on a polycarbonate membrane, and transepithelial electrical resistance (TEER) was improved with BCFA-enriched culture media [232]. BCFAs may promote the establishment of beneficial gut bacteria, as short BCFAs are incorporated into longer BCFAs present in bacterial membranes. This process of BCFA-enrichment of bacterial membranes has been demonstrated *in vitro* for *Ruminococcus* [232]. Although *Bifidobacterium* spp. [233] also incorporate long BCFA in their membranes, similar studies on the incorporation of short BCFA have not been performed. BCFA production from SRB provides a novel mechanism by which SRB may improve intestinal health.

A recent *in vitro* study showed that hemicelluloses of SRB bind both cholesterol and bile acids [234]. Increased fecal extraction of bile acids was shown in rats fed SRB [235], and to our knowledge, this is the first evidence for elevated bile acid and cholesterol excretion in human stool following increased SRB consumption. Sequestration of bile acids and cholesterol is generally considered beneficial and a primary clinical and dietary means for lowering blood lipid profiles [236]. However, we did not expect to see any significant changes in the plasma lipid parameters of participants in this study, because we investigated a healthy population with serum lipids in normal ranges.

Several beneficial phytochemicals were significantly increased in the stools of SRB participants compared with the baseline (Table 4.6). These included two rice bran phytosterols: cycloartenol and beta-sitosterol. Cycloartenol has been shown in animal studies to significantly reduce blood cholesterol and triglycerides [237] and also reduced 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation [238]. Additionally, in its trans-ferulate form, cycloartenol has anti-carcinogenic activity [239]. Beta-sitosterol scavenges free radicals and has shown potential as an anti-cancer drug through changed expression of beta-catenin and proliferating cell nuclear antigen (PCNA) [240]. Other increased phytochemicals include phenolics, namely hydrocinnamic acid, benzoic acid and phenylacetic acid. These compounds are all part of the phenylalanine metabolism pathway in KEGG [241]. Combinations of various derivatives of all three of these compounds in the form of fecal water extracts have previously been found to be anti-inflammatory by decreasing COX-2 activity [242]. While increased stool metabolites are not indicative of elevated systemic or host tissue levels of these compounds, they do have the potential to exert effects locally for the modulation of intestinal inflammation. The ability of this pilot intervention to detect significant increases in nine potentially SRB-derived metabolites (Table 4.6) underscores the potential for SRB intervention to modify the stool metabolome.

Of those metabolites significantly changed from the baseline, eleven are possibly of microbial origin (Table 4.6). The associated pathways for these metabolites suggest the following primary substrates, including: phenylalanine, primary bile acids and inositol phosphate. In addition, microorganisms produce beta-sitosterol and cycloartenol from a primary phytosterol gamma-oryzanol, which is known to be present in SRB [243]. Inositol phosphate can be produced by intestinal microbiota from diet-derived phytates, and previous research suggests this potential for phytate degradation [244]. The largest metabolite change noted in the current

study was indole-2-carboxylic acid, which showed a mean 12% increase with SRB consumption. This indole derivative is a known component of brown rice, but indole derivatives are also microbial metabolites resulting from protein degradation, particularly the amino acid tryptophan [245]. In addition, diverse phenolic compounds associated with or bound to dietary fiber, such as ferulic and other hydroxycinnamic acids, are converted by gut microbiota to a few of the same metabolites [246,247]. These phenolic metabolites include phenylacetic and benzoic acids, which were significantly increased with SRB supplementation in the current study. Taken together, these findings suggest that microbial fermentation of SRB in the gut may shape the resulting metabolites and their metabolic activity.

Conclusions

Previous research efforts characterizing SRB have largely focused on nutrient contents, phytochemicals (e.g., antioxidants) and the effects of consumption for the prevention and management of major chronic diseases. However, SRB modulation of the ~100 trillion microorganisms in the human gastrointestinal tract and alteration of their metabolic activities may result in the production of chemicals that confer its reported bioactivity. Despite the expected high level of inter-individual variation in the microbiota and metabolome, this pilot study demonstrated that SRB intake of 30 g/day changes stool bacterial populations after two and four weeks and results in the significant alteration of multiple plant- and microbe-derived stool metabolites. Additionally, several target outcome measures for larger clinical trials with SRB were identified. For example, SRB-associated increases in BCFA production that may reduce gut permeability and encourage the growth of *Bifidobacterium* should be confirmed. Other metabolites targeted for quantification in future studies include colonocyte-feeding SCFA, microbiota-

modulating secondary bile acids, anti-inflammatory SRB phytochemicals and indole-2-carboxylic acid as a candidate SRB intake biomarker in stool. This research emphasizes the value of pilot trials in confirming the feasibility and acceptability of the SRB intervention and targeting appropriate outcome measures prior to conducting research in a larger cohort. Understanding which organisms create bioactive SRB metabolites will be critical to achieving gastrointestinal disease prevention outcomes in people. Considering the microbial metabolism of SRB in humans will also improve our ability to advance its utility for improved intestinal health and the prevention of major chronic diseases.

Figures

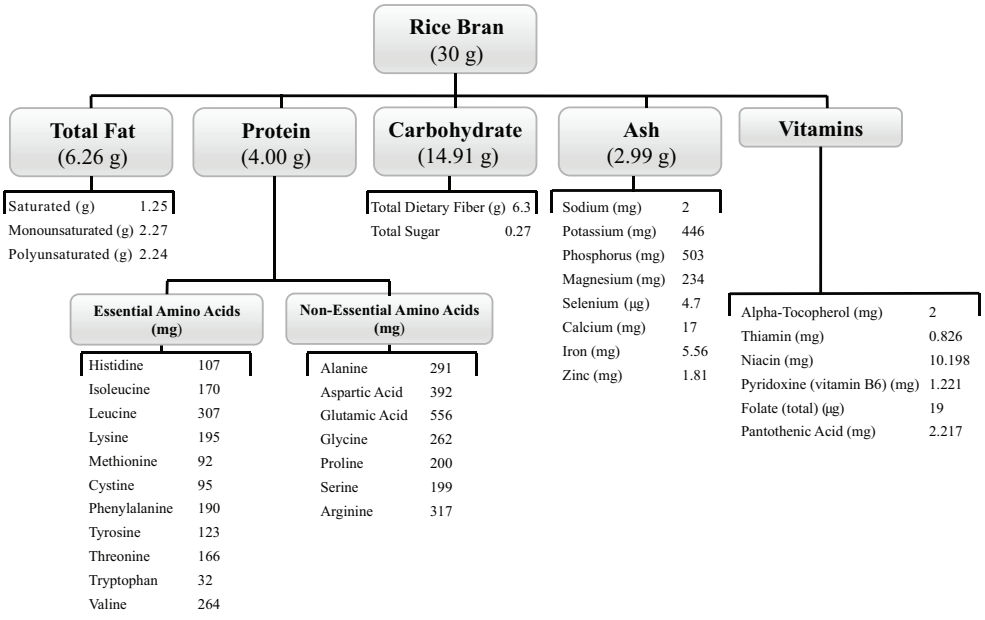


Figure 4.1. Nutrient composition of 30 g of heat-stabilized rice bran (SRB) [38]. The carbohydrate portion also includes unlisted starch.

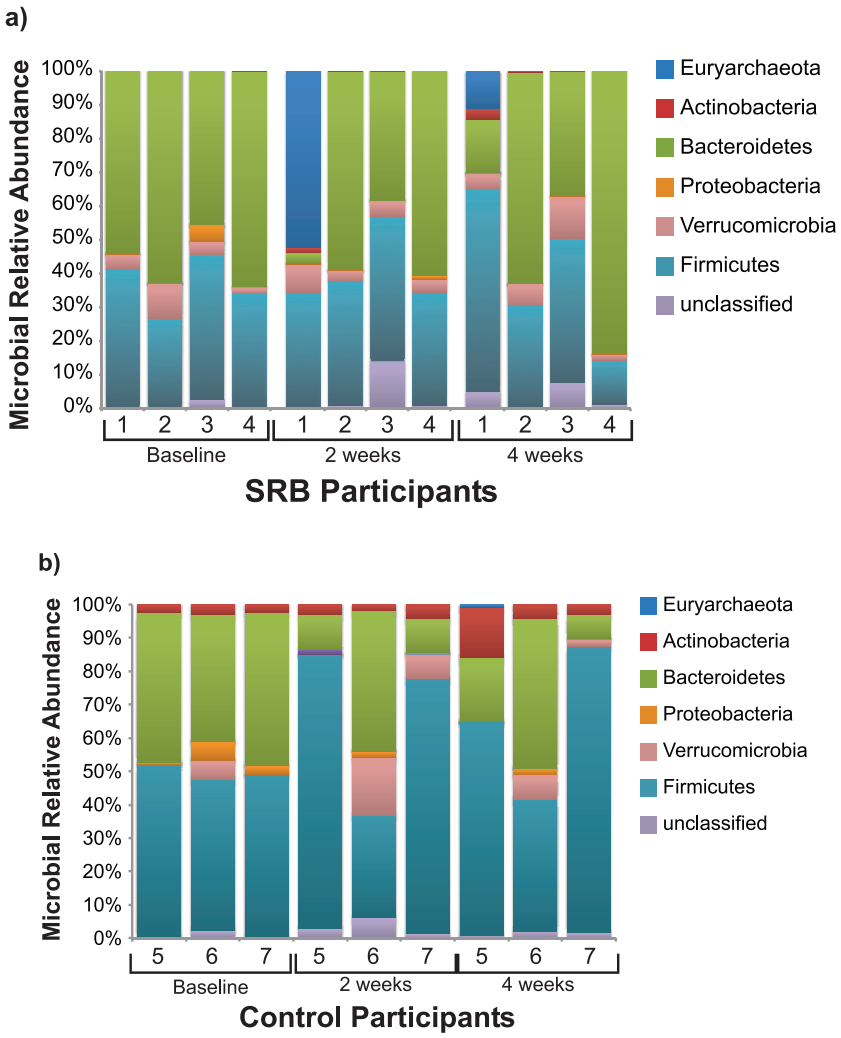


Figure 4.2. Phyla-level bacterial composition of stool samples for individual participants at baseline, two weeks and four weeks (**a**) with SRB and (**b**) without SRB.

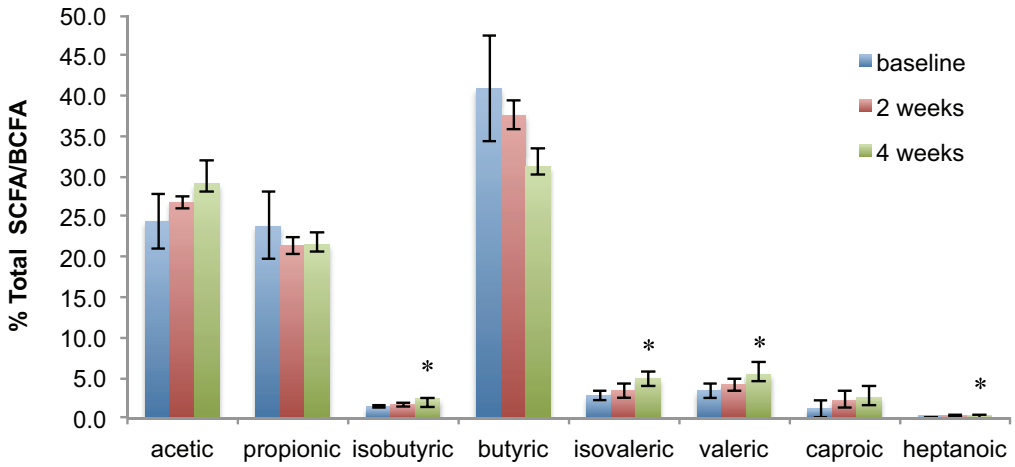


Figure 4.3. Proportional levels of SCFA and branched chain fatty acids (BCFA) in stool of SRB participants.

Tables

Table 4.1. Baseline characteristics of study participants.

Characteristic	Control (*n* = 3)	Rice Bran (*n* = 4)
Age (years) [a]	42.3 ± 21.7	42.8 ± 15.6
Sex		
Males (%)	2 (67%)	0 (0%)
Females (%)	1 (33%)	4 (100%)
BMI (kg/m^2) [a]	28.9 ± 6.9	22 ± 1.7
Total cholesterol [a] (mg/dL)	187 ± 57.2	197 ± 54.6
LDL [a] (mg/dL)	118 ± 50.3	127 ± 40.9
HDL [a] (mg/dL)	44 ± 12.6	54.3 ± 17.6
Triglycerides [a] (mg/dL)	125.7 ± 80.0	80 ± 35.0
Fruit intake (X servings/day) [b]		
$0 \leq X \leq 2$	2	3
$X > 2$	1	1
Vegetable intake (X servings/day) [b]		
$0 \leq X \leq 2$	1	1
$X > 2$	2	3
Grain intake (X servings/day) [b]		
$0 \leq X \leq 4$	2	4
$X > 4$	1	0

[a] Values are presented as the mean ± the standard deviation; [b] from the first three-day dietary food log.

Table 4.2. Total calories and macronutrient intake at the two-week and four-week time points for each study group *.

Dietary Intake	Control		Rice Bran	
	Week 2	Week 4	Week 2	Week 4
Calories (kcal)	2015.3 ± 325.0 (2186.4)	2047.8 ± 265.6 (2099.1)	2052.9 ± 410.3 (1940.6)	1925.3 ± 335.5 (1791.4)
Protein (g)	81.7 ± 13.7 (80.1)	77.6 ± 17.9 (77.6) [b]	86.3 ± 14.0 (85.3)	68.9 ± 9.9 (71.1) [b]
Carbohydrates (g)	264.6 ± 54.0 (290.8)	267.6 ± 53.0 (277.3) [a]	253.0 ± 46.4 (243.9)	255.6 ± 58.3 (241.4) [a]
Fat (g)	67.1 ± 13.9 (72.4)	74.6 ± 12.3 (81.0) [b]	79.8 ± 16.6 (74.2)	75.4 ± 13.3 (74.3) [a]
Fiber (g)	24.2 ± 3.0 (22.8) [a]	23.5 ± 8.0 (19.4) [b]	36.0 ± 7.5 (35.7) [a]	32.4 ± 5.6 (31.9) [b]

[*] Values are presented as the mean ± SD (median). Medians are included, since ranks were compared in the analysis; [a] Significance ($p \leq 0.05$) between the control and rice bran groups at time point; [b] Significance ($p \leq 0.05$) at Week 4 compared to Week 2 for the specific diet.

Table 4.3. Stool bacterial diversity at baseline (time = 1) and 4 weeks (time = 3) during intervention for both SRB and control participants.

Group	time	nseqs	coverage	S_{obs}	S_D	H'	E_H
SRB1	1	450	0.89	73	7.70	2.74	0.64
SRB2	1	450	0.85	114	15.37	3.46	0.73
SRB3	1	450	0.90	125	8.41	3.05	0.63
SRB1	3	450	0.90	113	7.66	3.11	0.66
SRB2	3	450	0.90	212	23.71	3.93	0.73
SRB3	3	450	0.88	133	20.68	3.82	0.78
pvalue			0.90	0.72	0.45	0.57	0.48
Control1	1	450	0.83	212	34.91	4.24	0.79
Control2	1	450	0.89	128	15.22	3.49	0.72
Control3	1	450	0.86	171	26.70	4.06	0.79
Control1	3	450	0.93	116	8.06	2.93	0.62
Control2	3	450	0.93	205	13.23	3.51	0.66
Control3	3	450	0.89	129	17.76	3.52	0.73
pvalue			0.16	0.02	0.31	0.04	0.09

S_{obs} = Total number of OTUs detected in a sample, S_D = Inverse Simpson Index
H' = Shannon Index, E_H = Shannon Evenness Index

Table 4.4. Percent change from baseline for bacterial operational taxonomic units (OTUs) that were significantly more abundant in the stool of individuals consuming SRB at two or four weeks.

Closest Hit in Database	2 weeks	*q*-Value	4 weeks	*q*-Value
Methanobrevibacter smithii	1201.00%	<0.001	210.73%	<0.001
Paraprevotella clara	352.87%	<0.001	156.71%	<0.001
Ruminococcus flavefaciens	128.49%	<0.001	79.02%	<0.001
Dialister succinatiphilus	86.59%	<0.001	57.47%	<0.001
Bifidobacterium sp.	2.79%	1.000	50.29%	0.003
Clostridium glycolicum (*Clostridium* cluster XI)	0.00%	1.000	40.71%	0.042
Barnesiella intestinihominis	277.35%	<0.001	66.31%	0.050
Anaerostipes caccae	90.09%	<0.001	69.63%	0.483
Ruminococcus bromii	66.77%	<0.001	29.47%	1.000

Table 4.5. Short chain and branched chain fatty acids identified from targeted GC-MS analysis at baseline, 2 weeks and 4 weeks in SRB participants.

	Control (n=3)			Rice Bran (n=4)		
SCFA	**Baseline**	**Week 2**	**Week 4**	**Baseline**	**Week 2**	**Week 4**
Acetic Acid	18.48 ± 1.82 (18.74)[a,b,d]	24.98 ± 5.15 (26.58)[a]	24.37 ± 2.55 (23.60)[b]	24.46 ± 6.26 (24.74)[d]	26.83 ± 2.28 (27.09)	29.11 ± 5.34 (29.31)
Propionic Acid	16.39 ± 2.42 (15.47)[d]	15.63 ± 3.13 (14.54)[d]	16.39 ± 3.75 (17.37)[d]	23.83 ± 7.55 (23.32)[d]	21.37 ± 1.30 (21.36)[d]	21.54 ± 3.10 (20.28)[d]
Isobutyric Acid	2.08 ± 0.57 (2.42)[d]	1.98 ± 1.04 (1.99)	1.42 ± 0.59 (1.06)[d]	1.38 ± 0.41 (1.53)[b,d]	1.71 ± 0.14 (1.72)[c]	2.38 ± 0.41 (2.44)[b,c,d]
Butyric Acid	44.98 ± 2.25 (44.29)	43.25 ± 5.97 (41.68)	45.36 ± 6.24 (43.83)[d]	40.93 ± 12.17 (38.72)[b]	37.57 ± 4.18 (39.03)[c]	31.24 ± 4.60 (32.49)[b,c,d]
Isovaleric Acid	5.51 ± 1.70 (6.10)[d]	5.27 ± 3.64 (4.31)	3.50 ± 1.97 (2.44)	2.79 ± 1.08 (2.86)[b,d]	3.44 ± 0.62 (3.33)[c]	4.98 ± 1.78 (4.68)[b,c]
Valeric Acid	6.25 ± 2.14 (7.00)[d]	5.51 ± 2.80 (6.10)	4.61 ± 2.11 (4.56)	3.37± 1.59 (3.46)[d]	4.15 ± 1.81 (3.33)[a]	5.47 ± 2.60 (4.68)
Caproic Acid	5.78 ± 3.77 (7.64)	2.92 ± 1.93 (3.48)	4.06 ± 2.89 (4.53)	2.32 ± 2.27 (2.12)	2.99 ± 1.97 (3.60)	3.41 ± 2.23 (4.34)
Heptanoic Acid	0.54 ± 0.30 (0.43)[d]	0.45 ± 0.42 (0.25)	0.29 ± 0.24 (0.23)	0.27 ± 0.23 (0.21)[d]	0.50 ± 0.34 (0.31)	0.55 ± 0.32 (0.55)

[*] Values are presented as mean percentage of total SCFAs ± SD (median). Medians are included since ranks were compared in the analysis. Footnote key on following page.

[a] Significance ($p \leq 0.05$) between Baseline and Week 2 of diet group

[b] Significance ($p \leq 0.05$) between Baseline and Week 4 of diet group

[c] Significance ($p \leq 0.05$) between Week 2 and Week 4 of diet group

[d] Significance ($p \leq 0.05$) between Control and Rice Bran at timepoint

Table 4.6. Candidate stool metabolites that differ between the baseline and four weeks in SRB participants ($p \leq 0.01$). Known phytochemical and nutritional components of rice bran are marked with an asterisk (*).

Stool Metabolites	% change at 4 weeks	KEGG pathway
Amino acids and nucleosides		
Inosine	3.72%	Purine metabolism
Uridine	3.22%	Pyrimidine metabolism
Glutamic acid *	1.82%	Purine and pyrimidine metabolism
Glutaric acid	1.73%	Lysine degradation
Glycine *	−1.56%	Purine metabolism
Leucine *	−3.75%	Amino acid metabolism
Cholesterol and bile acids		
Cholest-8(14)-en-3-one	6.78%	N/A
Deoxycholic acid	2.69%	Secondary bile acid biosynthesis
Cholest5-en-3-ol-propionate	2.12%	N/A
Lithocholic acid	1.07%	Secondary bile acid biosynthesis
Cholesterol	0.51%	Steroid biosynthesis
Phytochemicals and phenolics		
Indole-2-carboxylic acid *	11.65%	N/A
Hydrocinnamic acid	4.31%	Phenylalanine metabolism
Alpha-tocopherol *	2.46%	Vitamin digestion and absorption
Benzoic acid	2.39%	Phenylalanine metabolism
Cycloartenol *	1.90%	Steroid biosynthesis
Pantothenic acid *	1.90%	Vitamin digestion and absorption
Phenylacetic acid	1.49%	Phenylalanine metabolism
Beta-sitosterol *	0.11%	Steroid biosynthesis

Table 4. *Cont.*

Stool Metabolites	% change at 4 weeks	KEGG pathway
	Lipids	
Myristic acid *	7.32%	Fatty acid biosynthesis
Caprylic acid	3.84%	Fatty acid biosynthesis
Lauric acid	3.03%	Fatty acid biosynthesis
Palmitic acid *	2.20%	Fatty acid biosynthesis
Stearic acid *	1.12%	Fatty acid biosynthesis
Azelaic acid	0.56%	N/A
Glycerol	0.55%	Galactose metabolism
Oleic acid *	0.15%	Fatty acid biosynthesis
Sebacic acid	−0.33%	N/A
2-Hexenedioic acid	−0.32%	N/A
Pentadecanoic acid	−1.90%	N/A
	Putative microbial metabolites	
Indole-2-carboxylic acid *	11.65%	N/A
Hydrocinnamic acid [a]	4.31%	Phenylalanine metabolism
Inositol monophosphate [a]	3.90%	Inositol phosphate metabolism
Phosphoric acid [a]	3.61%	Peptidoglycan synthesis
Deoxycholic acid	2.69%	Secondary bile acid biosynthesis
	Putative microbial metabolites	
Benzoic acid [a]	2.39%	Phenylalanine metabolism
Cycloartenol [a]	1.90%	Steroid biosynthesis
Phenylacetic acid [a]	1.49%	Phenylalanine metabolism
Stearic acid [a]	1.12%	Fatty acid biosynthesis
Lithocholic acid	1.07%	Secondary bile acid biosynthesis
Beta-sitosterol [a]	0.11%	Steroid biosynthesis
	Sugars [b]	
Maltose	−0.10%	Carbohydrate digestion
Ribose	−3.56%	Carbohydrate digestion
Glucose	−3.63%	Carbohydrate digestion

[a] These metabolites may possibly be of plant origin, but can also be derived from microbial metabolism or modification of larger plant compounds, such as dietary fiber phenolics; [b] Sugar metabolites result from a wide range of metabolic pathways and could be of host, plant or microbial origin.

CHAPTER 5: DIETARY SUPPLEMENTATION WITH RICE BRAN OR NAVY BEAN ALTERS GUT BACTERIAL METABOLISM IN COLORECTAL CANCER SURVIVORS[78]

Summary

Heat-stabilized rice bran and cooked navy bean powder contain a variety of phytochemicals that are fermented by colonic microbiota and may influence intestinal health. Dietary interventions with these foods are being explored for modulating colorectal cancer risk. A randomized-controlled pilot clinical trial investigated the effects of eating heat-stabilized rice bran (30g/day) or cooked navy bean powder (35g/day) on gut microbiota and metabolites (NCT01929122). Twenty-nine volunteers with a prior history of colorectal cancer consumed a study-provided meal and snack daily for 28 days and stool samples were collected at 0, 14, and 28 days. Volunteers receiving rice bran or bean powder showed increased gut bacterial diversity and altered gut microbial composition at 28 days compared to baseline. Supplementation with rice bran or bean powder increased total dietary fiber intake similarly, yet only rice bran intake led to a decreased Firmicutes:Bacteroidetes ratio and increased short chain fatty acids

[7] A version of this chapter is in press at *Molecular Nutrition and Food Research:* Sheflin, A.M., Borresen, E.C., Kirkwood, J.S., Boot, C.M., Whitney, A.K., Lu, S., Brown, R.J., Broeckling, C.D., Ryan, E.P., Weir, T.L. (2016) with title *Dietary Supplementation with Rice Bran or Navy Bean Alters Gut Bacterial Metabolism in Colorectal Cancer Survivors.* Supporting material will be available online.

[8] The third aim of this work was to describe changes that occur with SRB and NBP supplementation in CRC survivors and infer possible implications for intestinal health and is described in this chapter. A version of this manuscript is currently in press at Molecular Nutrition and Food Research Journal. My contributions to this publication included choosing key analyses and target metabolites, sample extractions, and metabolite quantitation. In addition, I was lead contributor on 454 data analysis, results interpretation, statistical analyses, and writing the paper. Supporting information submitted for consideration as part of the manuscript under review has been incorporated into the main flow of this chapter.

This work is licensed under a Creative Commons Attribution 4.0 International License. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited. To view a copy of this license, visit https://creativecommons.org/licenses/by/4.0/

(propionate and acetate) in stool after 14 days but not at 28 days. These findings support modulation of gut microbiota and fermentation by-products by rice bran and suggest that foods with similar ability to increase dietary fiber intake may not have equal effects on gut microbiota and microbial metabolism.

Introduction

Diet is a well-established risk determinant in the development of sporadic (non-hereditary) colorectal cancer (CRC), which is thought to make up as much as 95% of all cases of the disease [248]. Since CRC has the second highest mortality rate of all cancers and is the third most common cancer in the US, nutritional intervention for prevention of CRC has particular value to reduce disease recurrence and mortality [249,250]. A large body of research associates diets low in whole grains and fiber with increased risk of CRC [248] and diets enriched with legumes and whole grains with reduced risk [8,55,251-255]. The underlying protective mechanisms mediated by these foods are thought to involve both fiber and polyphenols. Fiber is composed of polysaccharides and provides structure to the plant while polyphenols defend against UV radiation, herbivores, and infection [256,257]. Upon consumption, fiber provides the major energy source for microbial fermentation in the colon, which in turn stimulates the catabolism of plant polyphenols. An increase in polyphenol bioavailability results from bacterial enzymes that depolymerize, deglycosylate, and hydrolyze complex polyphenols into smaller phenolic acids capable of crossing the intestinal barrier [258]. Given the importance of these microbial functions it is likely that modulation of the gut microbiota and their metabolic processes is an important mechanism by which whole grains and legumes contribute to CRC prevention.

A growing body of recent research focuses on the role of the gut microbiota in the CRC disease process [67]. Multiple studies report different gut microbial communities in individuals diagnosed with colorectal cancer (CRC) versus healthy individuals [63-66]. In fact, gut microbiota can play a role in either promotion or prevention of CRC through modulation of the inflammatory process due to close contact with host colonic mucosa [68]. Furthermore, microbial-produced metabolites, such as short chain fatty acids (SCFA), modulate host intestinal barrier function [209,259], immune response [210,211], and inflammatory state. Tumor formation [133,212] and proliferation [213] are also directly affected by microbial metabolites of host dietary compounds. Changes in gut commensal microbiota associated with consumption of whole grains and legumes are not well defined, yet are crucial for understanding the contribution to CRC prevention.

Martínez et al. noted altered gut bacterial diversity, and changes in the Bacteroidetes:Firmicutes ratio and abundance of *Blautia spp.* due to consumption of unidentified non-starch components of whole grain brown rice flakes by healthy adults [26]. We previously demonstrated that dietary supplementation of 30g/day of heat-stabilized rice bran (SRB) selectively stimulated some bacterial groups including *Bifidobacterium spp*. and *Ruminococcus spp*. in a pilot human cohort with no history of CRC. In addition, increased stool branched chain fatty acids (BCFA) and polyphenol metabolites were associated with SRB supplementation [260]. Research featuring rice bran fermentation in an *in vitro* canine microbiome model has also revealed increases in anti-inflammatory short chain fatty acids (SCFA) [36]. Studies investigating effects of dry bean consumption on gut microbiota are limited to animal models and have identified limited effects [28,29] on gut microbial composition, but functional changes have not previously been investigated to our knowledge. Our pilot study in healthy adults with

no history of CRC [48] also failed to identify any changes to gut microbial composition with (35g/day) cooked navy bean powder (NBP) consumption for 28 days (data not published). Taken together, this previous research suggests while SRB and NBP are both high fiber foods, they do not induce similar effects on gut microbiota. Therefore, studying these two foods in conjunction offers a unique opportunity to explore the influence of dietary fiber on gut microbiota and their activities.

The influence of SRB or NBP supplementation on human gut microbial dynamics relevant to risk for CRC remain poorly understood. The present study is an exploratory pilot dietary intervention to investigate the potential for supplementation with SRB or NBP to alter gut microbiota and associated metabolites in CRC survivors. Our previous pilot study in a healthy cohort with no history of CRC [260] suggested that these dietary interventions are well tolerated in healthy adults with no history of CRC. So, the main objectives in the current study were to investigate changes in gut microbial composition, potential genetic functions, and resulting microbial metabolites following a one-month dietary supplementation in a cohort of healthy CRC survivors. Other research has demonstrated an association of CRC with gut microbial dysbiosis [67], suggesting that modulation of gut microbiota could be important in CRC prevention. Therefore, the goal of the current exploratory pilot study is to evaluate gut microbial response to SRB and NBP in CRC survivors. These data will be used to form hypotheses for future work regarding the long-term potential for SRB and NBP supplementation to prevent CRC recurrence.

Material and methods

Participants, study design and sample collection

A total of 37 CRC survivors, more than four months post cancer treatment (e.g. chemotherapy or radiation), were recruited and 29 completed the dietary invention study (Table 5.1). The pilot study was a four-week, randomized-controlled, single-blinded design with three treatment arms. All participants were randomly assigned to receive 30g of SRB, 35g of NBP or a macronutrient-matched control and consumed one study-provided meal and snack daily that included these additional ingredients. The study protocol was approved by the Colorado State University (CSU) Research Integrity and Compliance Review Office and the University of Colorado Health-North (UCH-North) Institutional Review Board (CSU protocol #: 09-1530H, and UCH-North IRB #: 10-1038). All participants provided written informed consent prior to enrollment. Participant recruitment, exclusion criteria, study design, and sample collection is described in detail in Borresen *et al.* [261].

This study analysis includes analysis of stool samples collected at time points 0 (baseline), 14 and 28 days for a total of three samples collected during the intervention. Stool samples were self-collected by participants and transported to the lab within 24 hours of each study visit and immediately stored at -20°C until further processing. Stool samples were homogenized, and three subsamples were collected with sterile cotton swabs. DNA was extracted from all samples using MoBio Powersoil DNA extraction kits (MoBio, Carlsbad, CA) according to the manufacturer's instructions and stored at -20°C prior to amplification steps. Samples with remaining material were aliquoted and stored at -80°C for metabolite analysis.

Pyrosequencing of the Bacterial Community

Sampling of human intestinal tissue is invasive and not without risk, therefore we followed the convention of using stool samples from study participants to classify and quantify gut bacteria at day 0 (baseline), day 14 and day 28. Library preparation and pyrosequencing of the V3-V5 region of the bacterial 16S rRNA gene was performed under contract at RTL (Lubbock, Texas). All 16S rRNA gene sequences were filtered for quality and processed with the open source bioinformatics tool mothur, Ver. 1.33 [214].

In order to normalize for differences in sample coverage, stool bacterial communities were randomly subsampled to equal the lowest number in a sample, or 1157 in this case. One sample had less than 800 reads and was excluded from the analysis for this reason. Sub-sampled data was used to calculate stool community diversity, including observed species richness (Sobs), estimated species richness (Chao) and Simpson's diversity (SD) using the mothur implementation of these calculators. All sequence data are publicly available through the Sequence Read Archive (SRA) under study Accession Number PRJEB14459, which is available at http://www.ebi.ac.uk/ena/data/view/ PRJEB14459.

Metagenome prediction with PICRUSt and STAMP

A synthetic metagenome was generated based on the observed 16S rRNA sequences for each diet intervention group [262]. The 16S rRNA sequences for each diet intervention group were used to predict the sample metagenome as described previously [263]. First, several scripts distributed with PICRUSt v 1.0.0 were used to prepare the data. OTUs with 99% similarity were clustered together using pick_closed_reference_otus.py and the OTU table was normalized for copies of the 16S rRNA gene using the normalize_by_copy_number.py script. This normalized OTU table was processed with the predict_metagenomes.py script and the predicted metagenome

was exported as to .biom format for analysis with the software package STAMP [264]. STAMP includes statistical and visualization tools that were used to identify differences in functional potential for the stool bacterial communities across the three diet intervention groups.

Stool Metabolite Quantification

Short chain fatty acids (SCFA)

One gram replicates of frozen stool samples were extracted for SCFA using 5mL acidified water (pH 2.5) as previously described [65] and spiked with 1mM of ethylbutyric acid which was used as an internal standard. Samples were separated and analyzed using a TG-WAXMS A column on an Agilent 6890 gas chromatograph equipped with an autosampler and FID detection. SCFAs were quantified by comparing peak areas to standard curves of commercial standards with acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, and valeric acid (Sigma, St. Louis, MO, USA). Study participants had significantly different variation in acetic acid at baseline via Wilcoxon rank sum test. Therefore, in order to normalize this difference, stool SCFA levels were presented as fold change (using peak area) at day 14 or 28 relative to day 0.

Bile acids and Phenolic acids

In order to assess changes in stool metabolites associated with the observed changes in microbiota with SRB at 14 and 28 days, quantification of bile acids and phenolic acids was performed on a subset of stool samples from SRB group participants (n=5). Stool samples were thawed to room temperature and 0.4-0.5 g each was mixed with sterile saline solution (0.9%) at a ratio of 1:10 (w/v). The mixture was vortexed to homogenize and centrifuged for 10 minutes at 1,000 x g at 4°C. Stool solutions were then diluted 1:2 with acetonitrile:water (1:2 v/v) and

vacuum filtered using a 0.22 μm nylon filter (Millipore, Billerica, MA, USA). LC-MS grade water was used for dilution. Filtered extract was again centrifuged for 25 minutes at 20,000 x g at 4°C to ensure clarity of the final extract before injection for Ultra-performance liquid chromatography tandem mass-spectrometry (UPLC-MS) analysis. For bile acid analysis, internal standards taurocholic acid-d5, deoxycholic acid-d4, and glycodeoxycholic acid-d4 were added to the extract at a final concentration of 0.67 μg/mL. For phenolic analysis, the extract was concentrated to 1/10 the original volume by drying under nitrogen, then re-suspended in 1:2 acetonitrile:water (v/v). LC-MS for the bile acid analysis was performed on a Waters Acquity UPLC coupled to a Waters Xevo TQ-S triple quadrupole mass spectrometer. Phenolic acid analysis was performed on an Agilent 6220 TOF MS using a dual-ESI source in negative ion polarity. Full details for these analyses have been detailed in the Supporting Information for this research.

Statistical analyses and data visualization

The student's t-test was used to compare stool bacterial richness, diversity and phyla composition at each time point to baseline. The SCFA and bile acid data were non-normally distributed. Therefore, the Wilcoxon signed rank sum test, a non-parametric test appropriate for repeated measures, was used to compare stool metabolite concentrations at each time point to baseline. To determine shifts in stool community structure, the mothur implementation of the analysis of molecular variance (AMOVA) test was used. To detect stool bacterial taxa differentiating each 14 and 28 day community from baseline, the online METASTATS [221] tool was used. STAMP was used to assess and visualize predicted functional differences in stool bacterial communities at 14 or 28 days compared to baseline for each supplemented diet [264].

In order to identify associations with potential biological relevance that could be used for hypothesis generation for future testing, a *p*-value less than 0.05 was considered for determining statistical significance in all analyses.

Results

Stool bacterial community diversity and composition

To investigate effects of supplementation with SRB or NBP on stool bacterial communities, richness (total number of OTUs detected), diversity (relative abundance of OTUs present), and composition were measured. Stool bacterial richness and diversity were calculated using Sobs (number of species observed), Chao1, and Simpson diversity indices. The Chao1 richness estimate is used to approximate the number of species potentially not detected due to incomplete sampling using bootstrapping. With SRB supplementation, richness and diversity in stool were not altered at day 14, but were significantly higher than baseline at day 28 (Fig. 5.1). Stool richness, as estimated by the Chao index, was also increased at day 28 but not day 14 with NBP supplementation (Fig. 5.1). No changes in richness or diversity were observed in samples from individuals consuming the control diet.

To assess potential shifts in stool community structure, principal coordinate analysis (PCoA) including all treatment time points was conducted. Consequently, AMOVA tests were used to compare stool communities at 14 and 28 days to baseline and revealed no significant changes in overall stool community structure in any of the dietary intervention treatment groups ($p < 0.05$).

Stool microbial composition on a phyla level showed significant changes with SRB. Overall, SRB supplementation increased abundance of *Bacteroidetes* and decreased the

abundance of *Firmicutes* resulting in a significantly lower *Firmicutes:Bacteroidetes* ratio (F:B ratio) at 14 days compared to baseline (Figure 5.2). Individuals varied in retention of the *Firmicutes:Bacteroidetes* ratio shift after 28 days of SRB consumption, resulting in no significant difference at 28 days relative to baseline. No changes in microbial composition at the phyla level were detected over time in the NBP or control diet groups.

For each treatment group, differentially abundant OTUs at 14 and 28 days relative to baseline were detected using the software package metastats [265]. No OTUs differed from baseline at either timepoint in the control group. In the NBP group, two OTUs at day 14 and five OTUs at day 28 differentiated the microbial community from baseline (Table S2). At 14 days, *Bacteroides fragilis* decreased and an unclassified *Lachnobacterium* increased in mean abundance relative to the NBP baseline microbial community. The reduction in mean abundance of *Bacteroides fragilis* persisted at 28 days and an unclassified *Clostridium* and *Anaerostipes* also had lower mean abundance with NBP. In addition, two OTUs had increased mean abundance at 28 days with NBP including an unclassified *Lachnospira* and *Coprococcus.* In the NBP group, four OTUs at day 14 and two OTUs at day 28 differentiated the microbial community from baseline (Table S2).

With SRB at 14 days, an unclassified *Ruminococcus* and unclassified *Ethanoligenens* were decreased in mean abundance relative to the baseline SRB microbial community. Conversely, an unclassified *Coprococcus* and *Bacteroides ovatus* were increased in mean abundance at 14 days. *Bacteroides ovatus* showed the second largest increase of any microorganism in this study with a 20-fold increase relative to baseline mean abundance. In addition, eight OTUs were newly detected and eleven OTUs were no longer detected as differential members at14 days compared to the baseline bacterial community (Table S2). The

number of OTUs discriminating the bacterial community at 28 days from baseline with SRB was larger than at 14 days (69 versus 23 OTUs). In addition, the largest increase in any single OTU occurred at 28 days with a 24-fold increase in *Lachnobacterium* relative to baseline mean abundance (Table S2).

Estimating change in functional potential of stool bacteria

The software package PICRUSt was used as a predictive tool to infer the content of the genome of each bacterium in a community as identified by a 16S rRNA sequencing. This genome was then correlated with a specific functional category as assigned in the Kyoto Encyclopedia of Genes and Genomes (KEGG) for understanding high-level functions of the gut microbial community. Differences in genetic function across treatments and time points in KEGG functional categories can be found using the software package STAMP [264]. Although not a perfect substitute for metagenomic sequencing, this approach allows for developing hypotheses related to the microbial community functions of the gut following dietary intervention.

Baseline differences between treatment groups were quite large and ultimately we were looking for responses to specific intervention foods. Therefore, we focused our analysis on a comparison of the 14 and 28-day time points from baseline for each food treatment group, and did not make comparisons between SRB and NBP groups. Several microbial metabolic functions were significantly enriched ($p< 0.05$) with SRB at 14 days compared to baseline (Figure 5.3). These functions included “Biosynthesis of unsaturated fatty acids”, “Phenylpropanoid biosynthesis”, “Other glycan degradation”, “Starch and sucrose metabolism”, “Streptomycin biosynthesis”, and “Sphingolipid metabolism” (Figure 3). The largest significant decrease in a

KEGG functional category was in genes for “Biosynthesis of unsaturated fatty acids”, which represent a significant portion of SRB composition [42]. No significant differences in KEGG functional categories were found at 28 days with SRB, or after 14 or 28 days for the control or NBP interventions when compared to baseline.

Targeted analysis of stool metabolites

SRB and NBP both contain highly fermentable carbohydrates that can be metabolized by gut bacteria to secondary byproducts such as SCFAs, particularly acetate, propionate and butyrate [224]. Given that functional genes for “Starch and sucrose metabolism” were enriched at 14 days with SRB, SCFAs were quantified to assess potential changes in stool microbial metabolites that resulted with SRB and NBP supplementation. No significant changes in stool SCFAs were observed at 14 or 28 days with the NBP intervention when compared to baseline (Figure 5.4a); however a large amount of inter-individual variation was observed in this group that could possibly obscure treatment effects. With SRB, no significant changes in stool butyric acid were observed at 14 or 28 days. However, both acetic and propionic acids were significantly increased with SRB at 14 days ($p < 0.05$); a change that did not persist at 28 days relative to baseline (Figure 5.4b). Acetic and propionic acids are byproducts of sugar, starch, and glycan fermentation and correspond with the observed enrichment in related metabolic genes at 14 days relative to baseline with SRB. Stool isobutyric and isovaleric acids were also measured based upon increases seen at 4 weeks with SRB consumption in our pilot study [260]. However, these BCFA were below detection limits in all participants using the current study participants and protocols.

Based on differences previously reported for bile acids at 28 days following SRB intake [260], we quantified deoxycholic acid, lithocholic acid, ursodeoxycholic acid, cholic acid, and chenodeoxycholic acid with the current study participants receiving SRB. Stool bile acid concentrations were highly variable across time points with SRB (Figure S1), with primary bile acids cholic acid and chenodeoxycholic trending downward at day 14. Microbial metabolites of polyphenols, including benzoic acid, 3,4-dihydrophenylacetic acid, phenylacetic acid, 3-phenylpropionic acid, 3-hydroxyphenylacetic acid, and 4-hydroxy-3-methoxycinnamic acid were also measured. None of the targeted polyphenol metabolites differed significantly in stool across time points or treatment groups ($p < 0.05$).

Discussion

Stool samples collected from this pilot human clinical dietary intervention trial of SRB or NBP supplementation in CRC survivors revealed changes in gut microbiota, associated metabolites, and predicted functions relative to baseline. None of these changes were observed in stool from participants in the control group. Since previous research has associated CRC with lower gut microbial richness and diversity [100], although somewhat inconsistently [67], we assessed changes to gut microbial richness and diversity with SRB and NBP intervention. In the present study, supplementation with NBP increased gut bacterial richness and SRB supplementation increased richness and diversity, both at 28 days. Our results with SRB do not concur with previous studies utilizing fermented rice bran or brown rice flakes, as these foods did not result in significant changes to participants' gut microbiota richness or composition [26,228]. However, consuming brown rice flakes (60g/day) did increase stool bacterial diversity after 4 weeks [26]. Since SRB is neither fermented nor refined prior to supplementation, this

difference could explain the shift in composition and increased species richness. In addition, our previous research in individuals with no history of cancer did not show increased stool bacterial diversity with NBP intake. Further research is necessary to clarify the ability of SRB and NBP supplementation to increase gut bacterial diversity.

Changes in gut microbial composition with SRB supplementation included a decrease in F:B ratio after 14 (2.7 down to 1.4), but not 28 days, relative to baseline. Average F:B ratio remained at 1.5 after 28 days, however the change at this time point was not significant due to large variation in individual response. Although an increased F:B ratio is commonly associated with a leaner phenotype in obesity studies , the role of F:B ratio in reference to CRC risk is not well characterized. However, previous studies have shown an increase in *Firmicutes* in tumor tissue relative to the intestinal lumen with CRC [63]. In addition, *Firmicutes* are also implicated in increased energy harvest [152], which may promote obesity and influence CRC risk. Therefore, reduction of *Firmicutes* may provide some protection against CRC. Both NBP and SRB increased total fiber in the participants' diets [261]; and while NBP increased gut bacterial richness, it did not change the F:B ratio. Furthermore, NBP had 2-5 differentiating bacterial taxa at 14 and 28 days compared to 20+ for SRB (Table S2). Since NBP participants had significantly lower *Firmicutes* at baseline than other groups ($p < 0.05$, ANOVA) it's possible that this initial lower abundance is the reason for less change in gut bacterial composition. It remains unclear whether F:B ratio, or gut bacterial richness or species have more relative importance for CRC risk. So, the relative importance for these findings for CRC chemoprevention cannot be confirmed.

The effect discrepancies between SRB and NBP suggests that the type of fiber-rich food introduced into the diet may be just as important as total fiber in the diet. Differences in the

dominant fiber components of SRB and NBP (i.e. arabinoxylan or xyloglucan) may explain differential effects detected herein on gut microbiota. The other phytochemicals such as polyphenols also influence gut microbial composition [266]. We did not see evidence of changes to phenolic acids or other polyphenol metabolite targets in stool with this research, but many forms of these phytochemicals exist beyond what we targeted to measure. Finally, while dry beans and other legumes are typical components of the diet, humans rarely consume SRB or large quantities of brown rice. Therefore, the shift in bacterial community composition may represent a classical ecological response to an environmental disturbance [267]. This is consistent with what appears to be a recovery of some of the baseline-level metabolic activities after 28 days. Mechanistic research identifying which specific components of high-fiber foods alter gut microbial dynamics and metabolic activities will be important for development of dietary therapies targeting the microbiome, including CRC chemoprevention strategies.

In addition to differences in composition of gut microbiota, the study comparing Native Africans (lower CRC risk) to African Americans (higher CRC risk) showed that Native Africans have higher stool SCFAs: acetate, butyrate, and propionate [142]. SCFAs play an important role in gut health by providing a primary energy source, reducing intestinal inflammation and regulating appetite and fat metabolism [268]. Supplementation with SRB increased both acetate and propionate at 14 days relative to baseline, a change that did not persist at 28 days. Acetate and propionate play complex roles in the gut metabolic environment. Propionate is implicated in glucose production and inhibition of cholesterol production from acetate in the liver [269]. While the increase in SCFAs at 14 days is promising for SRB ability to improve overall gut health, more research is necessary to determine what factors influence persistence of increased SCFAs.

We did not find changes in SCFA, bile acids or phenolic acids in this study with NBP at 14 or 28 days, which is consistent with findings of other research [10].

Multiple changes in gut microbiota were associated with the increased acetate and propionate with SRB at 14 days. An increase of greater than 20-fold in *Bacteroides ovatus* in addition to an increase in *Lachnospira spp.* occurred at 14 days with SRB supplementation. While little is known about *Lachnospira spp.* function in gut ecology, recent research has revealed that *B. ovatus* has a specific enzymatic toolkit that makes it uniquely suited to degradation of glycans [270]. The glycan, arabinoxylan, is a dominant constituent of the hemicellulose fraction of rice bran fiber [271]. Primary polysaccharide degraders, like *B.ovatus,* can release monosaccharides from cellulose and hemicellulose for further metabolism by a wide variety of gut commensals via glycolytic pathways. For example, *Lachnobacterium spp.,* which increased after 28 days, are unable to break down these primary polysaccharides but instead primarily utilize the monosaccharide byproducts of polysaccharide degradation. Pyruvate and oxaloacetate are primary intermediaries of gut fermentation of plant fiber to SCFA metabolites via glycolysis. *Blautia spp.* utilize the Wood-Ljungdahl pathway to produce acetic acid from pyruvate and were significantly more abundant (13-fold) in stools of the SRB group after 28 days relative to baseline. Propionate is a major fermentation product of *Bacteroides spp.* and *Coprococcus spp.* via the succinate and acrylate pathways respectively [272]. Independently of changes in stool SCFA concentrations, genes for “Streptomycin Biosynthesis” were enriched at day 14, which could also play a role in shaping the community at this time point since bacterial species vary in their ability to tolerate this bacteriocide.

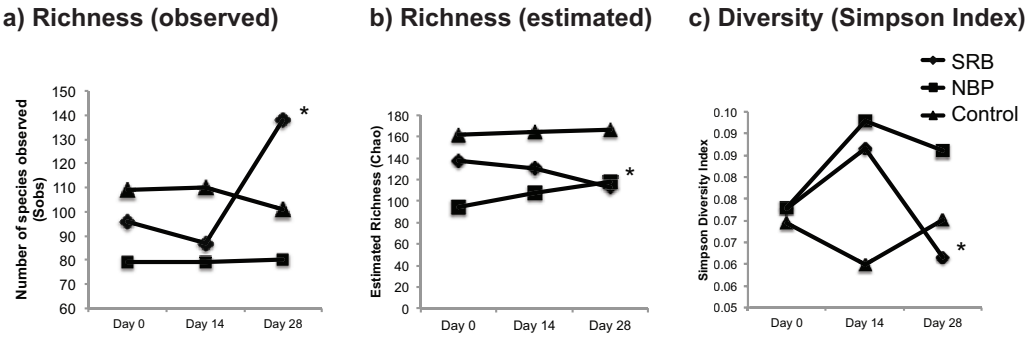
Concluding remarks

Previous research has suggested that microbial metabolism of food components during digestion plays a role in potential promotion or chemoprevention of CRC [59,133,212,213]. Consumption of high-fiber foods, such as SRB and NBP, has been associated with decreased CRC risk [8,55,251-254], which may be a result of changes in gut microbiota and their activities. In this study, both SRB and NBP increased stool bacterial richness or diversity, important stool community characteristics that are reduced in CRC relative to healthy populations. This research with CRC survivors supports the findings of our previous study in a healthy cohort without a history of cancer, namely by showing SRB intake of 30 g/day changes stool bacterial populations after 14 days. The current CRC survivor cohort also showed beneficial increases in acetic acid and propionic acid at 14 days that did not persist to 28 days. Further research is needed to determine whether more lasting changes with SRB intervention are possible, perhaps with an increased dose or utilizing other rice cultivars.

Due to the difficulty in recruiting large numbers of CRC survivors to participate in research studies, the number of participants limits interpretation of this study. The outcomes of this research should be used to guide future larger studies designed to control for inter-individual variation. Furthermore, the need for CRC survivor participants did not allow for exclusion and randomization based upon weight status. As such, the number of overweight and obese participants (21 out of 29 total) prevents understanding potential outcomes of SRB and NBP supplementation in a non-overweight CRC survivor cohort. Despite these limitations, these findings support the merit of further investigations of NBP and SRB supplementation as dietary interventions for CRC control and prevention. In addition, these results underscore the

importance of tracking microbiota in conjunction with long-term host physiologic responses such as inflammation, tumor recurrence, and intestinal barrier function in future research.

Figures



	S_{obs}			Chao			SD		
	Day 0	Day 14	Day 28	Day 0	Day 14	Day 28	Day 0	Day 14	Day 28
Rice1	65	68	116	78.6	91.0	98.3	0.062	0.07	0.06
Rice2	104	82	99	157.1	102.0	80.1	0.098	0.07	0.08
Rice3	63	59	67	75.8	89.0	58.7	0.114	0.22	0.11
Rice4	84	NA	132	119.1	NA	117.9	0.061	NA	0.05
Rice5	89	114	158	112.2	195.7	129.9	0.049	0.06	0.04
Rice6	132	123	214	195.1	182.1	181.2	0.044	0.03	0.04
Rice7	137	74	177	224.1	122.3	122.3	0.081	0.08	0.05
Bean1	78	91	78	95.3	111.0	95.3	0.08	0.07	0.08
Bean2	104	109	104	117.1	146.0	117.1	0.06	0.05	0.06
Bean3	81	74	81	102.2	114.6	102.2	0.07	0.14	0.07
Bean4	63	63	63	67.0	97.2	67.0	0.08	0.08	0.08
Bean5	68	56	68	91.1	69.3	91.1	0.07	0.12	0.07
Ctrl1	133	148	145	174.1	233.6	230.6	0.03	0.02	0.03
Ctrl2	106	119	103	129.6	153.7	164.8	0.11	0.08	0.09
Ctrl3	96	106	85	114.5	147.6	95.5	0.06	0.04	0.04
Ctrl4	81	NA	111	127.5	NA	165.1	0.07	NA	0.04
Ctrl5	99	115	44	168.5	181.0	66.7	0.07	0.05	0.17
Ctrl6	116	68	88	206.6	130.0	242.4	0.07	0.11	0.07
Ctrl7	85	69	151	140.7	96.1	235.9	0.12	0.11	0.04
Ctrl8	140	133	77	228.7	200.5	128.7	0.04	0.03	0.08
Ctrl9	126	121	NA	167.1	177.4	NA	0.05	0.04	NA

Figure 5.1. Stool bacterial diversity at days 0, 14, and 28 for SRB, NBP and control participants. All samples were normalized to 1157 sequences for each participant. Sobs = actual number OTUs detected (richness);Chao = estimated number OTUs (richness); SD = Simpson Diversity Index (lower values reflect higher diversity with this index). The table below the graphs lists data for each participant. The asterisk (*) indicates $p < 0.05$ with student's t-test comparing time point to day 0.

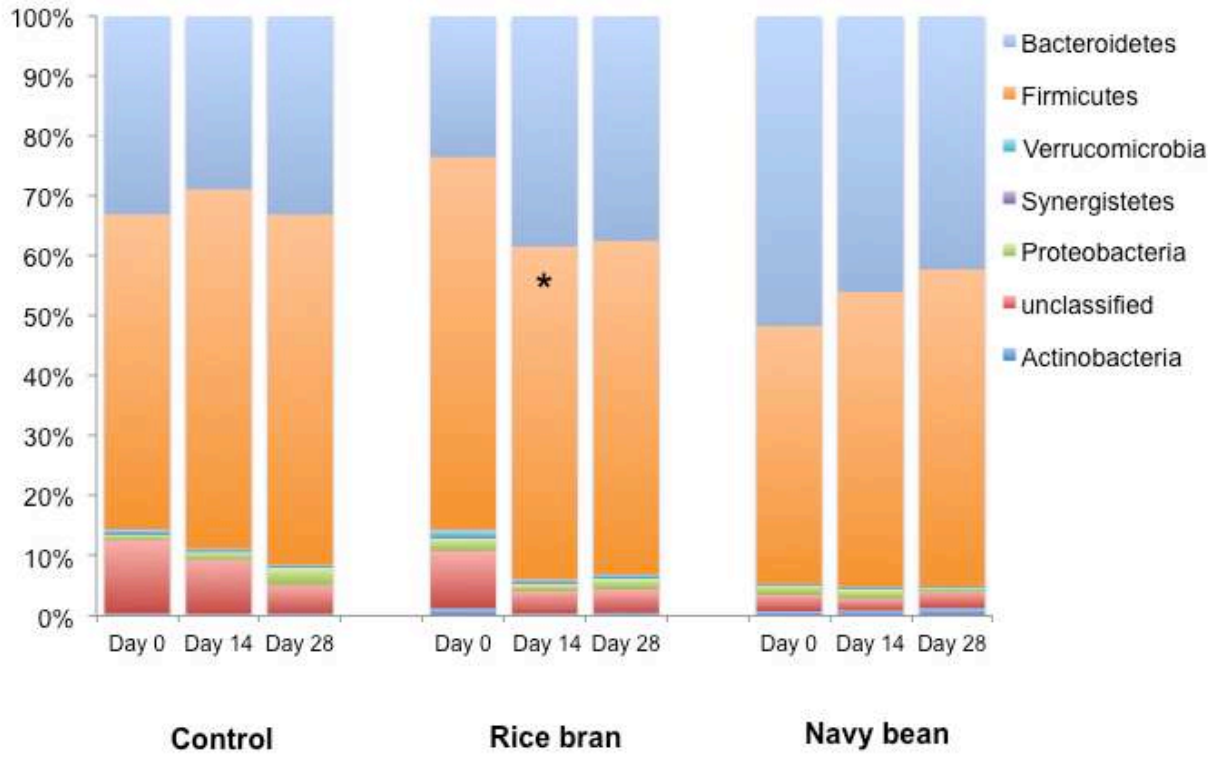


Figure 5.2. SRB is associated with decreased Firmicutes:Bacteroidetes ratio at 14 days relative to baseline, but not 28 days. The asterisk (*) indicates $p < 0.05$ with student's t-test comparing time point to day 0.

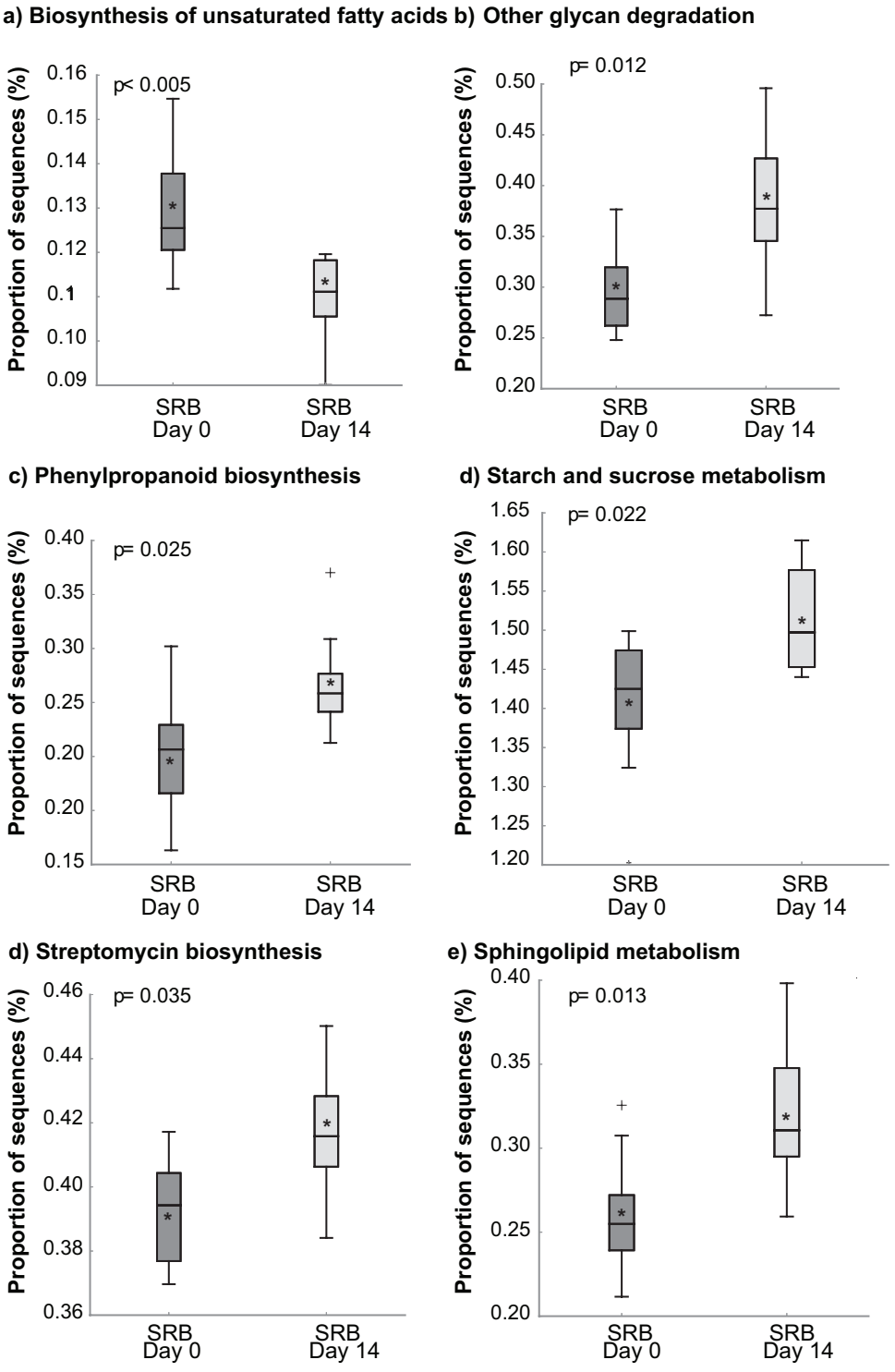


Figure 5.3. Predicted differences in stool bacterial functional categories (PICRUSt). Only KEGG categories found significant using STAMP analysis are shown, $p<0.05$.

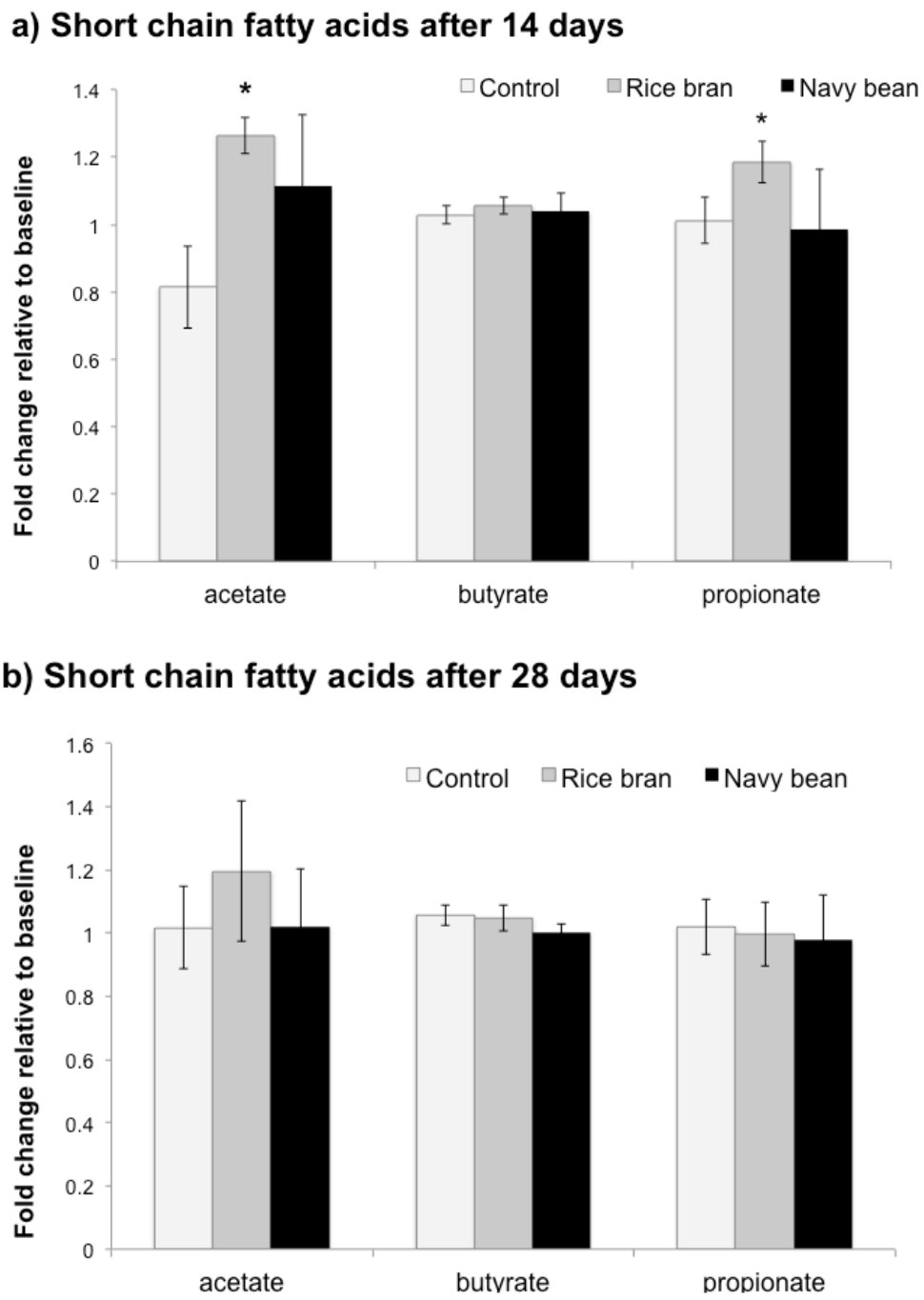


Figure 5.4. Changes in stool short chain fatty acids (SCFAs) shown in a) Day 14 and b) Day 28. The asterisk (*) indicates $p < 0.05$ with Wilcoxon rank sum test comparing fold change relative to baseline to control.

Tables

Table 5.1 Study participant characteristics.

ID	Group	Sex	Age	BMI
SRB1 [m]	SRB	FEMALE	58	24.1
SRB2 [m,s]	SRB	MALE	55	33.2
SRB3 [m,s]	SRB	MALE	70	34.5
SRB4 [m,s]	SRB	FEMALE	68	26.1
SRB5 [m,s,p,b]	SRB	MALE	53	31.1
SRB6 [m,s,p,b]	SRB	FEMALE	63	22.4
SRB7 [m,p,b]	SRB	MALE	51	32.5
SRB8 [s,p,b]	SRB	FEMALE	68	20.9
SRB9 [p,b]	SRB	FEMALE	68	32.3
C1 [m,s]	CONTROL	FEMALE	84	25
C2 [m,s]	CONTROL	FEMALE	61	31.3
C3 [m,s]	CONTROL	FEMALE	53	26.3
C4 [m,s,p]	CONTROL	MALE	75	31.4
C5 [m,s,p]	CONTROL	FEMALE	69	24.3
C6 [m,s,p]	CONTROL	MALE	62	28.5
C7 [m,s,p]	CONTROL	MALE	77	31.1
C8 [m,p]	CONTROL	FEMALE	62	22.3
C9 [s]	CONTROL	MALE	73	25.8
C10 [p]	CONTROL	FEMALE	39	25.6
NBP1[m]	NBP	MALE	57	24.7
NBP2 [m,s]	NBP	FEMALE	57	20.9
NBP3 [m]	NBP	FEMALE	53	35.3
NBP4 [m]	NBP	FEMALE	48	18
NBP5 [m]	NBP	FEMALE	60	25.7
NBP6 [s,p]	NBP	MALE	67	27.5
NBP7 [s,p]	NBP	FEMALE	70	32.8
NBP8 [s,p]	NBP	MALE	37	28.4
NBP9 [s,p]	NBP	FEMALE	59	46.4
NBP10 [p]	NBP	MALE	80	25.4

Sample included in [m] 454 pyrosequencing analysis of microbiota,
[s] stool SCFA's, [p] stool phenolic acids, and [b] stool bile acids analysis.

CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

The purpose of this research was to investigate the potential of supplementation with powdered, high-fiber staple foods; heat-stabilized rice bran (SRB) and navy bean powder (NBP), to favorably alter the intestinal environment for colorectal (CRC) prevention or control. Research methods utilized Next Generation Sequencing in combination with chromatography and mass spectrometry to profile changes in the gut microbial community, their metabolites and predicted metabolic functions. The results suggest that supplementation with SRB or NBP for 4 weeks alters gut microbiota and SRB also alters the stool metabolite profile making SRB a good candidate for further research on potential for CRC chemoprevention.

Across all three studies a large amount of inter-individual variation in microbiota composition was observed, which may be attributed to differences in genetics, long-term dietary habits, environmental factors and other influences outside of the control of study parameters. Crossover design studies, where each participant completes a control and treatment arm, can help normalize for variation between individuals and would be a superior design for dietary interventions that evaluate changes to gut microbiota. With both SRB and NBP, response of gut microbiota in CRC survivors was not consistent with the non-cancer cohort. These differences could be a result of the small sample sizes of each study population. However, these data may also suggest that previous health status may affect response to dietary intervention. The gut microbial community of study participants differed significantly at baseline across treatment groups in both studies. In addition, with regard to at least one microorganism, *Bacteroides ovatus*, response to SRB intervention was dependent on bacterial abundance in the participants at baseline. *B. ovatus* significantly increased at 2 weeks in all participants in whom it was detected

at baseline, but no increases were observed in participants in which these organisms were not initially detected (Fig. 6.1). These data suggest that the starting composition of the gut microbiota may also be a determinant in individual response to dietary intervention.

Study 1 compared stool samples from people with CRC diagnosis to healthy individuals in order to provide a basis of comparison for the pilot dietary interventions conducted in Study 2 and Study 3. Several stool microbial measures were analyzed including richness (number of species in a population), diversity (the richness and distribution of species), composition, and secondary metabolites. The data from Study 1 did not support a relationship of stool bacterial evenness or diversity with CRC, but this relationship has proved inconsistent across various studies [63-65,67,100]. However, these indices are sensitive to differences in sampling depth and sequence coverage, as well as inherent biases in the various algorithms used to calculate these parameters. With larger cohorts and increased sequencing depth the relationship between gut microbial diversity and CRC may be clarified and so it is important to consider effects on gut microbial richness and diversity with dietary change. In the pilot dietary interventions presented herein, no changes in microbial richness or diversity was observed in the non-cancer cohort. In CRC survivors, both NBP and SRB induced changes to gut microbial richness or diversity. In fact, the most remarkable effect of supplementation with NBP was to increase estimated stool bacterial richness (using Chao index) after 4 weeks. No significant changes to stool microbial metabolites or predicted bacterial functions occurred with NBP supplementation. With SRB supplementation, both richness and diversity of the gut microbial community increased at 4 weeks and this increase followed earlier changes at 2 weeks in Firmicutes:Bacteroidetes (F:B) ratio. There were also associated changes in predicted microbial functions and stool metabolites. Until the role of gut microbial diversity in the CRC disease process is better clarified and

described, it is difficult to interpret the importance of changes in gut bacterial diversity for potential CRC prevention with SRB or NBP supplementation.

Human gut microbiota are primarily (>90%) classified into two bacterial phyla, Bacteroidetes and Firmicutes and a higher ratio of these two bacterial groups (Firmicutes:Bacteroides) has been associated both with a high-fat dietary pattern and with obesity [273]. The F:B ratio has not shown a consistent relationship with CRC [63-65,274] and so its relevance to CRC progression is not clear. However, the two different phyla appear to prefer different physical niches in the gut environment. In patients with CRC, Bacteroidetes are most often associated with intestinal mucosa, whereas Firmicutes are more likely to be found in the intestinal lumen [163]. Long-term dietary pattern seems to be a reliable indicator as to which genera within the Bacteroidetes phyla will predominate. Native Africans tend to be dominated by the *Prevotella* genus while African Americans are predominated by *Bacteroides spp.* in previous studies looking at gut microbial composition. The compositional balance of these two phyla is known to shift in favor of Firmicutes with an obese phenotype, possibly due to their enhanced ability to harvest energy from the diet [273]. Recent studies examining the prebiotic activity of arabinoxylans, a constituent of SRB, suggest that this fiber component may rebalance a high F:B ratio induced by high fat diet [275]. Given the increased risk for CRC with obesity [276], continued monitoring of this ratio with dietary intervention is advised to gain a clearer picture of the role of dietary fats and microbial composition in CRC development.

Study 1 also defined some specific bacterial genera that were enriched in stool from healthy controls relative to CRC and included: *Bacteroides, Lachnospira,* and *Lachnobacterium*. Three species from the genus *Bacteroides* were enriched by 2-3 fold in stools of healthy individuals versus those with CRC. This finding corroborates other research showing higher

Bacteroides spp. in colon tissue from healthy people relative to CRC tumor tissue [180]. All three of these bacterial species were increased at either 2 or 4 weeks with either SRB or NBP supplementation in CRC survivors (Study 3). NBP supplementation increased an unidentified species from the genus *Lachnospira* by more than three-fold after 4 weeks of consumption. Since these bacteria rapidly ferment pectin and polygalacturonate, increases in *Lachnospira sp.* with NBP may be attributed to the arabinose-rich pectins and galacturonans present in cell walls of common bean (*P. vulgaris)* [277]. However, *Lachnospira spp.* cannot degrade other NBP components such as cellulose or xylans. *Lachnospira's* main fermentation byproducts (from glucose) include lactate, formate, acetate, ethanol, carbon dioxide, and some hydrogen gas [278]. Of these potential known byproducts, only stool acetate was measured in this research, however no significant changes in stool acetate were observed with NBP supplementation. Additional research in larger cohorts is encouraged to verify the relationship between NBP supplementation and increases in gut *Lachnospira spp.*

Also in the Lachnospiraceae family, *Lachnobacterium* were enriched in stool of healthy participants relative to those with CRC in Study 1 and increased in abundance with both NBP and SRB in the CRC survivor trial (Study 3). Supplementation with NBP increased an unidentified species of *Lachnobacterium* by nine fold after 2 weeks and another species increased by 24-fold with SRB after 4 weeks. Previous investigations targeting *Lachnobacterium* metabolism suggest that they do not metabolize complex polysaccharides present in SRB and NBP such as cellulose, xylans or pectins and that they produce only small amounts of acetate or butyrate [279]. Since *Lachnobacterium* primarily produce lactic acid from a diverse set of monosaccharides [279] and lactic acid was not measured in these studies, it is difficult to corroborate this metabolic pathway using data from this research. However, predicted functions

of gut bacteria were enriched for starch and sucrose metabolism after 2 weeks with SRB supporting that up-regulation of this metabolic pathway may have occurred. The role that *Lachnobacterium* and *Lachnospira* play in human gut ecology, health or disease is currently poorly understood, so it remains uncertain to what extent their increased abundance could prevent or influence proliferation of colorectal tumors.

In contrast to CRC survivors in Study 3, the non-cancer cohort in Study 2 had remarkably different responses to the dietary interventions with respect to gut microbial community composition. No changes in the *Bacteroides* genus as a whole or in its specific species were observed in non-cancer individuals. One possible explanation for this disparity is that there were different baseline communities and greater microbial community stability in the non-cancer cohort. We did note a twelve-fold increase in *Methanobrevibacter smithii,* observed at 2 weeks, which is consistent with possible cooperative degradation of recalcitrant fibers such as resistant starch [280]. Degradation of plant material, such as resistant starch, glycans, lignin and their subsequent polysaccharide byproducts frequently occurs via microbial cross-feeding due to the necessity for a large number of enzymatic functions to completely degrade the material [281]. This change was not observed in all SRB participants and standard error was relatively high (equal to the mean), therefore further research will need to confirm *M. smithii's* association with SRB supplementation. In addition, since sequencing primer choice imparts detection bias and different sequencing primers were used in Study 2 and Study 3, comparisons of bacterial compositions across these studies is limited. In particular, it was not possible to detect populations of *M. smithii* using the primer set from Study 3, so population increases observed in the non-cancer cohort could not be corroborated in CRC survivors. *Bifidobacterium* and *Ruminococcus* both increased in the non-cancer cohort with SRB at 2 and/or 4 weeks relative to

baseline and are known to play a role in resistant starch degradation. *R. bromii* is particularly key due to its role in releasing substrates that other gut bacterial species further process [272,280,282]. Taken as a whole, these data are consistent with potential cross-feeding relationships for SRB degradation in the gut microbial community. Further research could include more frequent sampling for a longer duration to clarify community dynamics that may occur with cooperative degradation of resistant starch, lignans, and glycans present in SRB.

Despite NBP supplementation providing more overall dietary fiber than SRB, it had less of an effect on gut microbiota and metabolites overall in both pilot dietary interventions. These results imply that total fiber intake may not be the most important dietary component for altering gut microbiota and their metabolism. The current research also supports previous findings by other research teams that suggest supplementation with rice-derived food products, such as whole grain rice cereal, induce more consistent and greater changes to gut microbiota than beans [26,28,29,36,260]. Possibly, the specific chemical composition of the fiber has more influence on gut microbial metabolism and colonic health than total dietary fiber intake. In order to decipher which fiber components are most influential in driving changes in the gut microbial community, metabolic byproducts and up-regulated enzymatic genes must be used to infer changing gut bacterial functions.

Study 3 (the CRC cohort) included a PICRUSt analysis providing predictions of functions that were enriched with SRB. After 2 weeks of SRB supplementation, the category of functional genes for 'other glycan degradation' was significantly enriched. Glycans are polysaccharides made up of sugar monomers (monosaccharides) bonded via β-1,4 linkages and include glucans which are composed of D-glucose monomers [283]. Glycans and glucans are common components of plant cell walls, including rice and beans [271,277,284], and component

monosaccharides are released during microbial fermentation in the gut. Genes for enzymes that degrade glycans and glucans to monosaccharides are included in the functional categories 'other glycan degradation' and 'starch and sucrose metabolism' were enriched at 2 weeks with SRB supplementation. Encoded enzymes included those important for mucin degradation (sialidases, fucosidases, and hexosaminidases) and those important for plant polysaccharide degradation (xylosidases, mannosidase, and beta-glucosidases) typically utilized in breaking down side-chain branches of glycans. Gut bacteria play a crucial role in digestion of plant-derived glycans, because human enzymes are not capable of cleaving the chemical bonds forming the component polysaccharides in these recalcitrant fibers [285].

Koropatkin et al. recently reviewed the mechanisms by which glycan metabolism shapes the human gut microbiota [286] where they suggest glycan availability is a major determinant of gut microbial composition and metabolism. They further propose dietary glycans as a potential means to manipulate the balance of species in the gut. This property is illustrated by the ability of human milk oligosaccharides, a diverse set of dietary glycans common in human milk but not other mammals [287]. Most of these oligosaccharides cannot be digested via human enzymes and have been proposed to selectively feed specific gut bacterial species and hence guide early infant gut colonization [288-290]. Glycans form branching structures that are shared not only by human milk oligosaccharides and hemicelluloses of plant cell walls, but also the protective layer of gut mucins. Mucins are made up of glycoproteins, which consist of glycan branches attached to a core protein backbone. Host and dietary glycans offer immense structural diversity and contain many different types of linkages requiring equally diverse bacterial enzymatic tools for degradation. As such, gut micobiota are highly variable in the types and numbers of glycans they

can degrade. In fact, the diversity present in mucin secretions may help protect the gut barrier from microbiota becoming too efficient at harvesting glycans from its surface [286].

Microbiota with broad ability to shift their metabolism to degrade a variety of dietary or host glycans have a survival advantage when dietary sources of glycans are depleted or changed. These microorganisms can be thought of as glycan-degrading 'generalists' and are capable of shifting their metabolism in the absence of dietary glycans to forage for glycans on the surface of host mucous secretions [291]. If glycan 'generalists' are present in the gut commensal community, a consistent supply of dietary glycans could prevent glycan foraging behavior and preserve the integrity of the protective mucins lining the gut surface. Marcobal et al. propose that co-evolution with gut commensals that prefer dietary glycans, when available, and exclude organisms that prefer intestinal mucin glycans represents an adaptive symbiotic relationship [292]. In fact, one study in mice suggests that genes involved in degradation of host mucins are upregulated when consuming a low fiber or Western diet relative to a polysaccharide-rich diet [293].

Detailed analysis of the dietary glycans present as hemicelluloses in rice bran and common bean have previously been conducted by other research teams [271,277,284]. Components of hemicellulose include xylan, xyloglucan, galactomannan and glucomannan and will vary according to botanical origin of the plant [286]. Rice bran and common bean differ substantially with regard to both xylan and xyloglucan content. Dry beans and other dicotyledonous plants are predominated by xyloglucans [277,284] whereas rice and other grasses are predominately xylans [294]. Chemical composition analysis of rice cell walls reveals that they are primarily made up of cellulose and arabinoxylan [271]. Arabinoxylan, like other xylans, is a linear polysaccharide made up of xylose sugars with β-D-(1—4)-linkages. *Bacteroides spp.*

produce necessary xylanases for degradation of either xyloglucans or xylans [295]. Salyers et al. tested 10 *Bacteroides* species and three had the ability to use xylan as a substrate. Xylan degraders identified in the Salyers et al. study included *Bacteroides fragilis* supsp*. a.*, and *B. vulgatus* as well as *B. ovatus* and *B. eggerthii,* which were increased with SRB supplementation in our CRC survivor cohort. *Bacteroides ovatus* has been shown to particularly target xylans with enzymes genetically encoded in two polysaccharide utilization loci (PULs) [286].One previous study in mice supplemented with wheat-bran suggests that arabinoxylans may be helpful in restoring high-fat-diet-induced shifts in F:B ratio [275]. Results from our pilot intervention with SRB in CRC survivors showed similar decreases in F:B ratio, although the persistence of this effect varied between individuals, resulting in a mean non-significant change at4 weeks. The F:B ratio does not play a clear role in the CRC disease development process, but it is implicated in increased energy harvest and obesity [296], which is a known CRC risk factor [276].

The decreased F:B ratio in CRC survivors supplemented with SRB was partly driven by increases in two xylan-degrading *Bacteroides spp.; B. ovatus* increased 20-fold at 2 weeks and *B. eggerthii* was newly detected at 4 weeks. *Bacteroides* are Gram-negative rod-shaped bacteria and mainly starch degraders, but some species also ferment amino acids. Major fermentation products of *Bacteroides* are acetate and propionate [297], which were significantly enriched in stools of SRB consuming individuals after 2 weeks in the CRC survivor pilot study. Increased acetate and decreased butyrate with CRC versus without was also noted in Study 1. Decreases in all three SCFA: acetate, propionate, and butyrate were noted when comparing stool samples from African Americans to native Africans, who experience lower rates of CRC. Increased SCFA are generally deemed beneficial to colonic health, but increased acetate has previously

been associated with CRC [298]. Given acetate's role in promoting proliferation in normal intestinal crypt cells [299], it's possible that this mechanism could also play a role in CRC progression.

Both acetate and propionate are proposed to play a physiological role in carbohydrate and lipid metabolism [300]. They also play important roles related to gastrointestinal blood flow [301], motility [302] and electrolyte balance [303]. Acetate may serve to exclude some enteric pathogens [304] and circulates to the liver where it may be utilized in adipogenesis [305]. Propioniate also circulates to the liver, but may help lower cholesterol by inhibiting its synthesis [305]. Sleeth et al. suggest that the decline in fermentable fiber intake post-Industrial Revolution could play a role in the obesity epidemic [268]. This argument is primarily justified via the role that SCFA, acetate and propionate in particular, play in energy homeostasis through acting on the G-protein coupled protein receptor free fatty acid receptor 2 (FFAR2; formerly GPR43). Experiments conducted in *ffar2* knockout mice demonstrate that increases in acetate and propionate stimulate peptide YY (PYY) and glucagon-like peptide (GLP)-1 secretion and enhance FFAR2 signaling [306]. Increased GLP-1 secretion with acetate and propionate has also been noted in humans [307,308]. Ongoing research into therapeutics for enhanced GLP-1 secretion is showing promise for potential benefits in subjects with diabetes and obesity [309]. Given the association of obesity, diabetes and increased CRC risk, enhanced GLP-1 secretion with acetate and propionate increases may play a protective role against CRC occurrence.

Increases in acetate and propionate, and associated *B. ovatus,* observed in CRC survivors (Study 3), were transient and not sustained after 4 weeks with SRB supplementation. Further research is necessary to understand reasons for the transient nature of acetate and propionate increases, but one potential reason could be an unreported decrease in study compliance that led

to an overall decrease in SRB exposure. Another potential reason could be microbial community dynamics enabled new species to populate the gut that produce alternative SRB fermentation byproducts. The increase in stool bacterial diversity observed at 4 weeks also supports the idea of community succession where a founder bacterial species modifies the environment making it more favorable for multiple additional species to thrive (Fig 5.1).

In addition to microbial metabolites of fiber, byproducts of protein degradation were significantly increased in two of the three research studies. Study 1 identified increased branched chain fatty acids (BCFA) with CRC, which is characteristic of protein putrefaction with high-meat, low-fiber diets and may increase CRC risk [213,310]. Increases in BCFA were also observed with SRB supplementation in the non-cancer pilot intervention after 4 weeks, but not in the CRC pilot intervention. Given the implications of BCFA with increased CRC risk, these metabolites should continue to be monitored with SRB supplementation to clarify the association. Potential increases in colonic BCFA are not often considered when encouraging protein consumption for its ability to reduce appetite and sustain satiety after eating [311]. Furthermore, both men and women over the age of 20 in the US over-consume dietary protein [312]. Recommendations of increased fiber intake should be considered as an alternative dietary means for sustained satiety, because fermentation of fiber to SCFA can provide a similar reduction in appetite via FFAR2 signaling. In addition, more than 90% of children and adults in the US do not meet daily fiber recommendations [313].

Since SRB and NBP differ chemically in more ways than just fiber composition, it is also possible that another component of SRB, such as polyphenols, is driving changes in gut microbial dynamics. Stool samples were enriched with potential byproducts of polyphenols with SRB supplementation in Study 2 (non-cancer cohort) (Table 4.6), but not in Study 3 (CRC

survivors). A possible explanation for this disparity may be a reduced capacity to metabolize polyphenols in CRC survivors. The targeted metabolite analysis conducted in Study 3 may also have failed to capture changes in metabolites that were not specifically monitored. Further research should continue to quantify metabolites of polyphenols to clarify the importance of these metabolites with SRB supplementation.

Since bile acids are known to alter the gut microbial community [59] and CRC risk [314], we also quantified these metabolites. Changes in secondary bile acids with SRB were inconsistent and are therefore difficult to interpret. Secondary bile acids lithocholic acid and deoxycholic acid increased after 4 weeks with SRB in Study 2 (non-cancer cohort) but not Study 3 (CRC survivor cohort). Ursodeoxycholic acid (UDCA), which was higher in healthy people relative to those with CRC in Study 1, showed substantial increases in some participants in Study 3 (CRC survivor cohort) but was not significant overall. An increase in stool secondary bile acids is typically associated with a high fat diet, but SRB can also bind and excrete these pro-carcinogenic byproducts [315]. So, while the importance of secondary bile acids with SRB supplementation remains unclear after this research, these microbial metabolites may prove to be important in future research. Continued measurement of these metabolites with SRB supplementation is encouraged, particularly if the cause of variation in individual response can be determined.

Observations across these three studies include differences in overall gut microbial ecology and metabolism between CRC patients versus healthy adults and with supplementation of SRB or NBP. These differences can be examined in the context of the several recent hypotheses on how gut microbial dysbiosis may contribute to CRC initiation and progression reviewed in Chapter 2. The ‘alpha-bug’ and ‘passenger-driver’ hypotheses both theorize that the

CRC disease process is initiated with establishment of a keystone pathogen, such as ETBF. Highly diverse and evenly distributed gut microbial communities are in a state of eubiosis and are resistant to pathogen establishment due to commensal acidification and competitive exclusion. Our results showed an increase in SCFA production after 2 weeks with SRB supplementation that reflects gut microbial degradation of SRB fiber into acidic byproducts. Using SRB as a dietary tool to increase acidic byproducts in the colonic environment may play an important role in pathogen exclusion and deserves further research attention. Next the hypotheses theorize that an inflammatory event disrupts the ability of the gut microbial community to exclude pathogens. However, it remains unclear whether a keystone pathogen first establishes and initiates an inflammatory process or if host inflammation allows for pathogenic rearrangement of the microbial community. One could speculate based on these study results that host inflammation may result from a low fiber diet that forces some species of gut bacteria to utilize gut mucins for energy in the absence of an alternative substrate such as dietary glycans. Degradation of the gut mucin layer, would expose the colonic tissues to digestive byproducts, enterotoxins, and pathogen interactions. Once bacteria have access to intestinal tissues, DNA can be damaged via bacterial production of chromosome-breaking factors, also known as the bystander effect. This exposure would likely increase risk of CRC initiation and tumor formation and would further alter the colonic environment to favor 'passenger' bacteria such as *Akkermansia muciniphila* that take advantage of tumor mucous secretions. Our observation of increased *Akkermansia muciniphila* in CRC patients versus healthy adults supports this hypothetical fit of study data with the reviewed hypotheses.

An important comment with regard to the pilot dietary intervention research herein acknowledges that both potential positive and potential negative outcomes were observed. In our

quest to improve diet for modulation of gut microbial metabolism and disease prevention, it is important to resist the urge to either vilify or sanctify specific foods. Nearly all foods, like the NBP and SRB used in this study, are composed of a wide variety of chemical components that induce an equally varied and complex microbial response that includes functions and metabolites that work in opposition for affect on total host health. The variety of microbial activities underscores their importance and the need to consider gut microbial functions in a whole diet context. The combined effects of diet and byproducts of microbial metabolism will influence inflammation, DNA stability, oxidative stress and cell cycle regulation that ultimately will either prevent or promote CRC formation or progression. However, it is unrealistic to expect that any food will exert only beneficial effects on host or microbial physiology.

Summary and future directions

Increasing dietary fiber intake, in the form of whole-grain cereals and legumes, is recommended for cancer prevention and to support intestinal health. However, much remains to be learned about the mechanisms by which this health improvement occurs. By understanding gut fermentation of plant fibers, more consistent results and more effective dietary therapies can be attained. Plant fiber is a complex substance with varied components and can be vastly different depending on the food's botanical origin. The results of this research suggest that designing effective dietary therapies will require moving beyond the term 'dietary fiber' and recognizing the individual qualities of its chemical constituents. Particular attention must be paid to glycan-containing high-fiber foods since the organisms that degrade glycans typically also employ mucin-degrading metabolic pathways.

While much work has been done *in vitro* to investigate the ability of specific *Bacteroides* organisms to switch between metabolism of dietary glycans to host mucin glycan degradation, less is known about the *in vivo* effects of dietary glycan flux. Future research should include short and long-term studies that investigate changes to gut microbial glycan foraging behavior before, during and after provision of dietary glycans present in foods like SRB. Chronic underfeeding of specific biochemically versatile gut microorganisms may prove to play an important role in dysbiosis of the gut community and/or degradation of the protective mucus layer both of which have been implicated in the initiation and progression of CRC. However,these three studies collectively suggest potential for beneficial alteration of gut microbial composition with SRB and NBP supplementation. SRB, being rich in arabinoxylans, offers particular promise for preservation of protective gut mucins by sparing host glycans and also an improved stool metabolite profile consistent with CRC prevention.

Figures

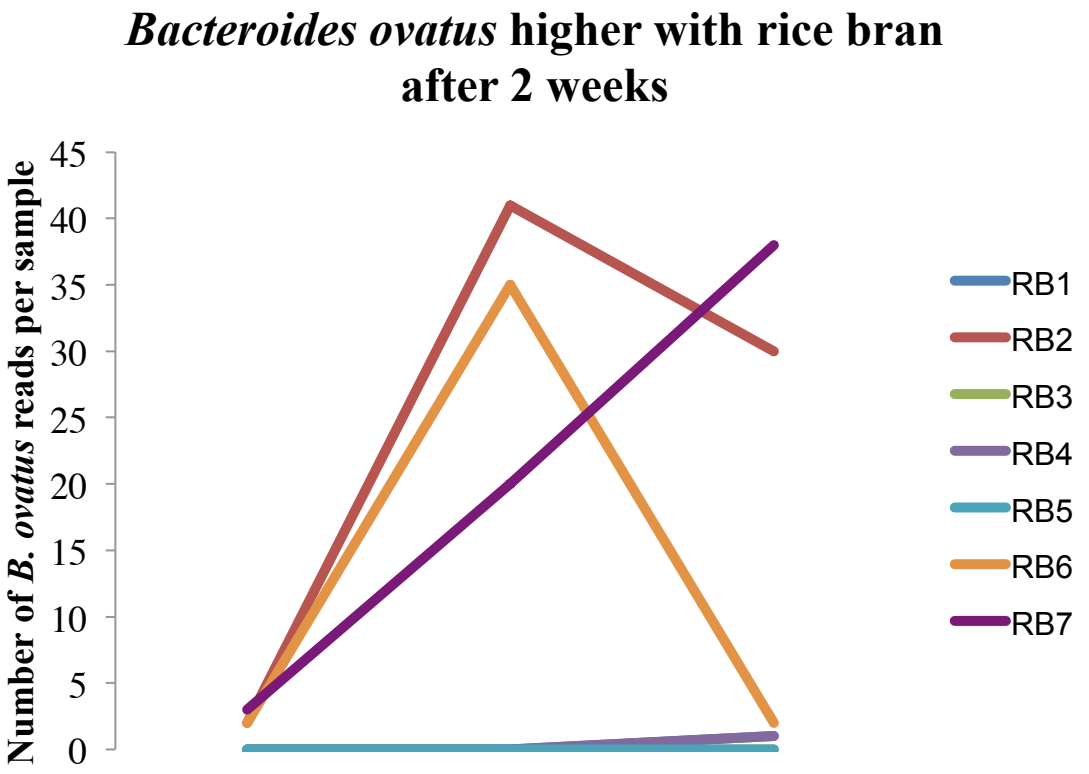


Figure 6.1 *Bacteroides ovatus* increases after 2 weeks with SRB supplementation.

REFERENCES

1. Conference, F.R.; Food; Nations, A.O.o.t.U. *Proceedings of the FAO Rice Conference: Rice is Life*. Food and Agriculture Organization of the United Nations: 2004.
2. Dasgupta, S.; Roy, I. Proceedings of the regional consultation on the promotion of pulses in Asia for multiple health benefits. **2016**.
3. Mitchell, D.C.; Lawrence, F.R.; Hartman, T.J.; Curran, J.M. Consumption of dry beans, peas, and lentils could improve diet quality in the US population. *Journal of the American Dietetic Association* **2009**, *109*, 909-913.
4. Committee, D.G.A. Scientific Report of the 2015 Dietary Guidelines Advisory Committee. *Washington (DC): USDA and US Department of Health and Human Services* **2015**.
5. Hayat, I.; Ahmad, A.; Masud, T.; Ahmed, A.; Bashir, S. Nutritional and health perspectives of beans (Phaseolus vulgaris L.): an overview. *Critical reviews in food science and nutrition* **2014**, *54*, 580-592.
6. Henderson, A.J.; Ollila, C.A.; Kumar, A.; Borresen, E.C.; Raina, K.; Agarwal, R.; Ryan, E.P. Chemopreventive properties of dietary rice bran: current status and future prospects. *Adv. Nutr.* **2012**, *3*, 643-653.
7. Park, Y.; Hunter, D.J.; Spiegelman, D.; Bergkvist, L.; Berrino, F.; van den Brandt, P.A.; Buring, J.E.; Colditz, G.A.; Freudenheim, J.L.; Fuchs, C.S. Dietary fiber intake and risk of colorectal cancer: a pooled analysis of prospective cohort studies. *Jama* **2005**, *294*, 2849-2857.
8. Schatzkin, A.; Mouw, T.; Park, Y.; Subar, A.F.; Kipnis, V.; Hollenbeck, A.; Leitzmann, M.F.; Thompson, F.E. Dietary fiber and whole-grain consumption in relation to colorectal cancer in the NIH-AARP Diet and Health Study. *The American journal of clinical nutrition* **2007**, *85*, 1353-1360.
9. Center, M.M.; Jemal, A.; Smith, R.A.; Ward, E. Worldwide variations in colorectal cancer. *CA: a cancer journal for clinicians* **2009**, *59*, 366-378.
10. Perera, T.; Young, M.R.; Zhang, Z.; Murphy, G.; Colburn, N.H.; Lanza, E.; Hartman, T.J.; Cross, A.J.; Bobe, G. Identification and monitoring of metabolite markers of dry bean consumption in parallel human and mouse studies. *Mol. Nutr. Food Res.* **2015**, *59*, 795-806.
11. Center, M.M.; Jemal, A.; Ward, E. International trends in colorectal cancer incidence rates. *Cancer Epidemiology Biomarkers & Prevention* **2009**, *18*, 1688-1694.
12. O'Keefe, S.J.; Chung, D.; Mahmoud, N.; Sepulveda, A.R.; Manafe, M.; Arch, J.; Adada, H.; van der Merwe, T. Why do African Americans get more colon cancer than Native Africans? *J. Nutr.* **2007**, *137*, 175S-182S.
13. Fuchs, C.S.; Giovannucci, E.L.; Colditz, G.A.; Hunter, D.J.; Stampfer, M.J.; Rosner, B.; Speizer, F.E.; Willett, W.C. Dietary fiber and the risk of colorectal cancer and adenoma in women. *New England Journal of Medicine* **1999**, *340*, 169-176.
14. Howe, G.R.; Benito, E.; Castelleto, R.; Cornée, J.; Estève, J.; Gallagher, R.P.; Iscovich, J.M.; Deng-ao, J.; Kaaks, R.; Kune, G.A. Dietary intake of fiber and decreased risk of cancers of the colon and rectum: evidence from the combined analysis of 13 case-control studies. *Journal of the National Cancer Institute* **1992**, *84*, 1887-1896.

15. Norat, T.; Chan, D.; Lau, R.; Aune, D.; Vieira, R. The associations between food, nutrition and physical activity and the risk of colorectal cancer. *WCRF/AICR Systematic Literature Review Continuous Update Project Report. London: World Cancer Research Fund/American Institute for Cancer Research* **2010**.
16. Bednar, G.E.; Patil, A.R.; Murray, S.M.; Grieshop, C.M.; Merchen, N.R.; Fahey, G.C. Starch and fiber fractions in selected food and feed ingredients affect their small intestinal digestibility and fermentability and their large bowel fermentability in vitro in a canine model. *J. Nutr.* **2001**, *131*, 276-286.
17. Lattimer, J.M.; Haub, M.D. Effects of dietary fiber and its components on metabolic health. *Nutrients* **2010**, *2*, 1266-1289.
18. Macfarlane, G.T.; Macfarlane, S. Bacteria, colonic fermentation, and gastrointestinal health. *J. AOAC Int.* **2012**, *95*, 50-60.
19. Aune, D.; Chan, D.S.; Lau, R.; Vieira, R.; Greenwood, D.C.; Kampman, E.; Norat, T. Dietary fibre, whole grains, and risk of colorectal cancer: systematic review and dose-response meta-analysis of prospective studies. *Bmj* **2011**, *343*, d6617.
20. Zeng, H.; Lazarova, D.L.; Bordonaro, M. Mechanisms linking dietary fiber, gut microbiota and colon cancer prevention. *World J Gastrointest Oncol* **2014**, *6*, 41-51.
21. Vince, A.; McNeil, N.; Wager, J.; Wrong, O. The effect of lactulose, pectin, arabinogalactan and cellulose on the production of organic acids and metabolism of ammonia by intestinal bacteria in a faecal incubation system. *Br. J. Nutr.* **1990**, *63*, 17-26.
22. Macfarlane, G.T.; Macfarlane, S. Fermentation in the human large intestine: its physiologic consequences and the potential contribution of prebiotics. *Journal of clinical gastroenterology* **2011**, *45*, S120-S127.
23. Liu, R.H. Whole grain phytochemicals and health. *Journal of Cereal Science* **2007**, *46*, 207-219.
24. Gordon, A.R.; Cohen, R.; Crepinsek, M.K.; Fox, M.K.; Hall, J.; Zeidman, E. The third school nutrition dietary assessment study: Background and study design. *Journal of the American Dietetic Association* **2009**, *109*, S20-S30.
25. Kern, F.; Birkner, H.J.; Ostrower, V.S. Binding of bile acids by dietary fiber. *The American journal of clinical nutrition* **1978**, *31*, S175-S179.
26. Martínez, I.; Lattimer, J.M.; Hubach, K.L.; Case, J.A.; Yang, J.; Weber, C.G.; Louk, J.A.; Rose, D.J.; Kyureghian, G.; Peterson, D.A*., et al.* Gut microbiome composition is linked to whole grain-induced immunological improvements. *ISME J.* **2013**, *7*, 269-280, papers2://publication/doi/10.1038/ismej.2012.104.
27. Henderson, A.J.; Kumar, A.; Barnett, B.; Dow, S.W.; Ryan, E.P. Consumption of rice bran increases mucosal immunoglobulin A concentrations and numbers of intestinal Lactobacillus spp. *J. Med. Food* **2012**, *15*, 469-475.
28. Kerr, K.R.; Forster, G.; Dowd, S.E.; Ryan, E.P.; Swanson, K.S. Effects of dietary cooked navy bean on the fecal microbiome of healthy companion dogs. *PloS one* **2013**, *8*.
29. Queiroz-Monici, K.d.S.; Costa, G.E.; da Silva, N.; Reis, S.M.; de Oliveira, A.C. Bifidogenic effect of dietary fiber and resistant starch from leguminous on the intestinal microbiota of rats. *Nutrition* **2005**, *21*, 602-608.
30. Díaz-Batalla, L.; Widholm, J.M.; Fahey, G.C.; Castaño-Tostado, E.; Paredes-López, O. Chemical components with health implications in wild and cultivated Mexican common bean seeds (Phaseolus vulgaris L.). *J. Agric. Food Chem.* **2006**, *54*, 2045-2052.

31. Wiboonsirikul, J.; Kimura, Y.; Kanaya, Y.; Tsuno, T.; Adachi, S. Production and characterization of functional substances from a by-product of rice bran oil and protein production by a compressed hot water treatment. *Biosci., Biotechnol., Biochem.* **2008**, *72*, 384-392.
32. Devkota, S.; Chang, E.B. Nutrition, microbiomes, and intestinal inflammation. *Current opinion in gastroenterology* **2013**.
33. Kau, A.L.; Ahern, P.P.; Griffin, N.W.; Goodman, A.L.; Gordon, J.I. Human nutrition, the gut microbiome and the immune system. *Nature* **2011**, *474*, 327-336.
34. Shanahan, F. The colonic microbiota in health and disease. *Current opinion in gastroenterology* **2013**, *29*, 49-54.
35. Rycroft, C.; Jones, M.; Gibson, G.; Rastall, R. A comparative in vitro evaluation of the fermentation properties of prebiotic oligosaccharides. *Journal of Applied Microbiology* **2001**, *91*, 878-887.
36. Ogué−Bon, E.; Khoo, C.; McCartney, A.L.; Gibson, G.R.; Rastall, R.A. In vitro effects of synbiotic fermentation on the canine faecal microbiota. *FEMS Microbiol. Ecol.* **2010**, *73*, 587-600.
37. Eid, N.; Walton, G.; Costabile, A.; Kuhnle, G.G.; Spencer, J.P. Polyphenols, glucosinolates, dietary fibre and colon cancer: Understanding the potential of specific types of fruit and vegetables to reduce bowel cancer progression. *Nutrition and Aging* **2014**, *2*, 45-67.
38. Halliwell, B.; Zhao, K.; Whiteman, M. The gastrointestinal tract: a major site of antioxidant action? *Free radical research* **2000**, *33*, 819-830.
39. Stevenson, L.; Phillips, F.; O'sullivan, K.; Walton, J. Wheat bran: its composition and benefits to health, a European perspective. *Int. J. Food Sci. Nutr.* **2012**.
40. Ryan, E.P. Bioactive food components and health properties of rice bran. *Journal of the American Veterinary Medical Association* **2011**, *238*, 593-600.
41. Prasad, M.N. Health benefits of rice bran—A review. *J. Nutr. Food Sci.* **2011**, *1*, 108, doi:10.4172/2155-9600.1000108.
42. Kahlon, T.S. *Rice Bran: Production, Composition, Functionality and Food Applications, Physiological Benefits*. Taylor and Francis Group, LLC: Boca Raton: 2009.
43. Juliano, B.O. *Rice chemistry and quality*. Philippine rice research institute: 2003.
44. Camacho, L.M.; Gutiérrez, M.; Alarcón-Herrera, M.T.; Villalba, M.d.L.; Deng, S. Occurrence and treatment of arsenic in groundwater and soil in northern Mexico and southwestern USA. *Chemosphere* **2011**, *83*, 211-225.
45. Rahman, M.A.; Hasegawa, H.; Rahman, M.M.; Rahman, M.A.; Miah, M. Accumulation of arsenic in tissues of rice plant (< i> Oryza sativa</i> L.) and its distribution in fractions of rice grain. *Chemosphere* **2007**, *69*, 942-948.
46. Abedin, M.J.; Cresser, M.S.; Meharg, A.A.; Feldmann, J.; Cotter-Howells, J. Arsenic accumulation and metabolism in rice (Oryza sativa L.). *Environmental science & technology* **2002**, *36*, 962-968.
47. Hoseney, R.C. *Principles of cereal science and technology*. American Association of Cereal Chemists (AACC): 1994.
48. Borresen, E.C.; Gundlach, K.A.; Wdowik, M.; Rao, S.; Brown, R.J.; Ryan, E.P. Feasibility of increased navy bean powder consumption for primary and secondary colorectal cancer prevention. *Current nutrition and food science* **2014**, *10*, 112.

49. Champ, M.M.-J. Non-nutrient bioactive substances of pulses. *Br. J. Nutr.* **2002**, *88*, 307-319.
50. Cardador-Martínez, A.; Loarca-Piña, G.; Oomah, B.D. Antioxidant Activity in Common Beans (Phaseolus vulgaris L.) §. *J. Agric. Food Chem.* **2002**, *50*, 6975-6980.
51. Beninger, C.W.; Hosfield, G.L. Antioxidant activity of extracts, condensed tannin fractions, and pure flavonoids from Phaseolus vulgaris L. seed coat color genotypes. *J. Agric. Food Chem.* **2003**, *51*, 7879-7883.
52. Wu, X.; Beecher, G.R.; Holden, J.M.; Haytowitz, D.B.; Gebhardt, S.E.; Prior, R.L. Lipophilic and hydrophilic antioxidant capacities of common foods in the United States. *J. Agric. Food Chem.* **2004**, *52*, 4026-4037.
53. Cardador-Martinez, A.; Castano-Tostado, E.; Loarca-Pina, G. Antimutagenic activity of natural phenolic compounds present in the common bean (Phaseolus vulgaris) against aflatoxin B 1. *Food Addit. Contam.* **2002**, *19*, 62-69.
54. de Mejıa, E.G.; Castano-Tostado, E.; Loarca-Pina, G. Antimutagenic effects of natural phenolic compounds in beans. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* **1999**, *441*, 1-9.
55. Bennink, M. Consumption of black beans and navy beans (Phaseolus vulgaris) reduced azoxymethane-induced colon cancer in rats. *Nutr. Cancer* **2002**, *44*, 60-65.
56. Correa, P. Epidemiological correlations between diet and cancer frequency. *Cancer research* **1981**, *41*, 3685-3689.
57. Freudenheim, J.L.; Graham, S.; Horvath, P.J.; Marshall, J.R.; Haughey, B.P.; Wilkinson, G. Risks associated with source of fiber and fiber components in cancer of the colon and rectum. *Cancer research* **1990**, *50*, 3295-3300.
58. Reynoso-Camacho, R.; Ramos-Gomez, M.; Loarca-Pina, G. 10 Bioactive components in common beans (Phaseolus vulgaris L.). **2006**.
59. David, L.A.; Maurice, C.F.; Carmody, R.N.; Gootenberg, D.B.; Button, J.E.; Wolfe, B.E.; Ling, A.V.; Devlin, A.S.; Varma, Y.; Fischbach, M.A. Diet rapidly and reproducibly alters the human gut microbiome. *Nature* **2013**.
60. Clemente, J.C.; Ursell, L.K.; Parfrey, L.W.; Knight, R. The impact of the gut microbiota on human health: an integrative view. *Cell* **2012**, *148*, 1258-1270.
61. Meyerhardt, J.A.; Niedzwiecki, D.; Hollis, D.; Saltz, L.B.; Hu, F.B.; Mayer, R.J.; Nelson, H.; Whittom, R.; Hantel, A.; Thomas, J. Association of dietary patterns with cancer recurrence and survival in patients with stage III colon cancer. *Jama* **2007**, *298*, 754-764.
62. Almendingen, K.; Hofstad, B.; Vatn, M.H. Dietary habits and growth and recurrence of colorectal adenomas: results from a three-year endoscopic follow-up study. *Nutr. Cancer* **2004**, *49*, 131-138.
63. Marchesi, J.R.; Dutilh, B.E.; Hall, N.; Peters, W.H.; Roelofs, R.; Boleij, A.; Tjalsma, H. Towards the human colorectal cancer microbiome. *PloS one* **2011**, *6*, e20447.
64. Sobhani, I.; Tap, J.; Roudot-Thoraval, F.; Roperch, J.P.; Letulle, S.; Langella, P.; Corthier, G.; Van Nhieu, J.T.; Furet, J.P. Microbial Dysbiosis in Colorectal Cancer (CRC) Patients. *PLoS ONE* **2011**, *6*, e16393, 10.1371/journal.pone.0016393.
65. Weir, T.L.; Manter, D.K.; Sheflin, A.M.; Barnett, B.A.; Heuberger, A.L.; Ryan, E.P. Stool microbiome and metabolome differences between colorectal cancer patients and healthy adults. *PloS One* **2013**, *8*, e70803, doi:10.1371/journal.pone.0070803.

66. Zackular, J.P.; Baxter, N.T.; Iverson, K.D.; Sadler, W.D.; Petrosino, J.F.; Chen, G.Y.; Schloss, P.D. The Gut Microbiome Modulates Colon Tumorigenesis. *mBio* **2013**, *4*, e00692-00613.
67. Sheflin, A.M.; Whitney, A.K.; Weir, T.L. Cancer-Promoting Effects of Microbial Dysbiosis. *Curr. Oncol. Rep.* **2014**, *16*, 1-9.
68. Jobin, C. Colorectal cancer: looking for answers in the microbiota. *Cancer discovery* **2013**, *3*, 384-387.
69. Thomas, L.V.; Ockhuizen, T. New insights into the impact of the intestinal microbiota on health and disease: a symposium report. *Br. J. Nutr.* **2012**, *107*, S1-S13.
70. Consortium, H.M.P. Structure, function and diversity of the healthy human microbiome. *Nature* **2012**, *486*, 207-214.
71. Yang, Y.; Wang, X.; Huycke, T.; Moore, D.R.; Lightfoot, S.A.; Huycke, M.M. Colon Macrophages Polarized by Commensal Bacteria Cause Colitis and Cancer through the Bystander Effect. *Translational oncology* **2013**, *6*, 596.
72. Candela, M.; Turroni, S.; Biagi, E.; Carbonero, F.; Rampelli, S.; Fiorentini, C.; Brigidi, P. Inflammation and colorectal cancer, when microbiota-host mutualism breaks. *World J Gastroenterol* **2014**, *20*, 908-922.
73. Schwabe, R.F.; Jobin, C. The microbiome and cancer. *Nature Reviews Cancer* **2013**, *13*, 800-812.
74. Sears, C.L.; Garrett, W.S. Microbes, Microbiota, and Colon Cancer. *Cell host & microbe* **2014**, *15*, 317-328.
75. Zhan, Y.; Chen, P.-J.; Sadler, W.D.; Wang, F.; Poe, S.; Núñez, G.; Eaton, K.A.; Chen, G.Y. Gut microbiota protects against gastrointestinal tumorigenesis caused by epithelial injury. *Cancer research* **2013**, *73*, 7199-7210.
76. Winter, S.E.; Winter, M.G.; Xavier, M.N.; Thiennimitr, P.; Poon, V.; Keestra, A.M.; Laughlin, R.C.; Gomez, G.; Wu, J.; Lawhon, S.D. Host-derived nitrate boosts growth of E. coli in the inflamed gut. *Science* **2013**, *339*, 708-711.
77. Brenchley, J.M.; Douek, D.C. Microbial translocation across the GI tract. *Annual review of immunology* **2012**, *30*, 149.
78. Hajishengallis, G.; Darveau, R.P.; Curtis, M.A. The keystone-pathogen hypothesis. *Nature Reviews Microbiology* **2012**, *10*, 717-725.
79. Tjalsma, H.; Boleij, A.; Marchesi, J.R.; Dutilh, B.E. A bacterial driver–passenger model for colorectal cancer: beyond the usual suspects. *Nature Reviews Microbiology* **2012**, *10*, 575-582.
80. Grivennikov, S.I.; Wang, K.; Mucida, D.; Stewart, C.A.; Schnabl, B.; Jauch, D.; Taniguchi, K.; Yu, G.-Y.; Österreicher, C.H.; Hung, K.E. Adenoma-linked barrier defects and microbial products drive IL-23/IL-17-mediated tumour growth. *Nature* **2012**, *491*, 254-258.
81. Pimentel-Nunes, P.; Teixeira, A.L.; Pereira, C.; Gomes, M.; Brandão, C.; Rodrigues, C.; Gonçalves, N.; Boal-Carvalho, I. Functional polymorphisms of Toll-like receptors 2 and 4 alter the risk for colorectal carcinoma in Europeans. *Digestive and Liver Disease* **2013**, *45*, 63-69.
82. Richmond, A. NF-κB, chemokine gene transcription and tumour growth. *Nature Reviews Immunology* **2002**, *2*, 664-674.

83. Schwitalla, S.; Fingerle, A.A.; Cammareri, P.; Nebelsiek, T.; Göktuna, S.I.; Ziegler, P.K.; Canli, O.; Heijmans, J.; Huels, D.J.; Moreaux, G. Intestinal tumorigenesis initiated by dedifferentiation and acquisition of stem-cell-like properties. *Cell* **2013**, *152*, 25-38.
84. Zubair, A.; Frieri, M. Role of nuclear factor-κB in breast and colorectal cancer. *Current allergy and asthma reports* **2013**, *13*, 44-49.
85. Neufert, C.; Becker, C.; Türeci, Ö.; Waldner, M.J.; Backert, I.; Floh, K.; Atreya, I.; Leppkes, M.; Jefremow, A.; Vieth, M. Tumor fibroblast–derived epiregulin promotes growth of colitis-associated neoplasms through ERK. *The Journal of clinical investigation* **2013**, *123*, 1428.
86. Couturier-Maillard, A.; Secher, T.; Rehman, A.; Normand, S.; De Arcangelis, A.; Haesler, R.; Huot, L.; Grandjean, T.; Bressenot, A.; Delanoye-Crespin, A. NOD2-mediated dysbiosis predisposes mice to transmissible colitis and colorectal cancer. *The Journal of clinical investigation* **2013**, *123*, 700.
87. Shanahan, M.T.; Carroll, I.M.; Grossniklaus, E.; White, A.; von Furstenberg, R.J.; Barner, R.; Fodor, A.A.; Henning, S.J.; Sartor, R.B.; Gulati, A.S. Mouse Paneth cell antimicrobial function is independent of Nod2. *Gut* **2013**.
88. Nagi, R.S.; Bhat, A.S.; Kumar, H. Cancer: a tale of aberrant PRR response. *Frontiers in immunology* **2014**, *5*.
89. Pradere, J.; Dapito, D.; Schwabe, R. The Yin and Yang of Toll-like receptors in cancer. *Oncogene* **2013**.
90. Collins, D.; Hogan, A.M.; Winter, D.C. Microbial and viral pathogens in colorectal cancer. *The lancet oncology* **2011**, *12*, 504-512.
91. Buc, E.; Dubois, D.; Sauvanet, P.; Raisch, J.; Delmas, J.; Darfeuille-Michaud, A.; Pezet, D.; Bonnet, R. High prevalence of mucosa-associated E. coli producing cyclomodulin and genotoxin in colon cancer. *PloS one* **2013**, *8*, e56964.
92. Goodwin, A.C.; Shields, C.E.D.; Wu, S.; Huso, D.L.; Wu, X.; Murray-Stewart, T.R.; Hacker-Prietz, A.; Rabizadeh, S.; Woster, P.M.; Sears, C.L. Polyamine catabolism contributes to enterotoxigenic Bacteroides fragilis-induced colon tumorigenesis. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 15354-15359.
93. Kostic, A.D.; Chun, E.; Robertson, L.; Glickman, J.N.; Gallini, C.A.; Michaud, M.; Clancy, T.E.; Chung, D.C.; Lochhead, P.; Hold, G.L. < i> Fusobacterium nucleatum</i> Potentiates Intestinal Tumorigenesis and Modulates the Tumor-Immune Microenvironment. *Cell host & microbe* **2013**, *14*, 207-215.
94. Bongers, G.; Pacer, M.E.; Geraldino, T.H.; Chen, L.; He, Z.; Hashimoto, D.; Furtado, G.C.; Ochando, J.; Kelley, K.A.; Clemente, J.C. Interplay of host microbiota, genetic perturbations, and inflammation promotes local development of intestinal neoplasms in mice. *The Journal of experimental medicine* **2014**, *211*, 457-472.
95. Castellarin, M.; Warren, R.L.; Freeman, J.D.; Dreolini, L.; Krzywinski, M.; Strauss, J.; Barnes, R.; Watson, P.; Allen-Vercoe, E.; Moore, R.A. Fusobacterium nucleatum infection is prevalent in human colorectal carcinoma. *Genome research* **2012**, *22*, 299-306.
96. Geng, J.; Fan, H.; Tang, X.; Zhai, H.; Zhang, Z. Diversified pattern of the human colorectal cancer microbiome. *Gut Pathog* **2013**, *5*.
97. Flanagan, L.; Schmid, J.; Ebert, M.; Soucek, P.; Kunicka, T.; Liska, V.; Bruha, J.; Neary, P.; Dezeeuw, N.; Tommasino, M. Fusobacterium nucleatum associates with stages of

colorectal neoplasia development, colorectal cancer and disease outcome. *European Journal of Clinical Microbiology & Infectious Diseases* **2014**, 1-10.
98. Rubinstein, M.R.; Wang, X.; Liu, W.; Hao, Y.; Cai, G.; Han, Y.W. < i> Fusobacterium nucleatum</i> Promotes Colorectal Carcinogenesis by Modulating E-Cadherin/β-Catenin Signaling via its FadA Adhesin. *Cell host & microbe* **2013**, *14*, 195-206.
99. Png, C.W.; Lindén, S.K.; Gilshenan, K.S.; Zoetendal, E.G.; McSweeney, C.S.; Sly, L.I.; McGuckin, M.A.; Florin, T.H. Mucolytic bacteria with increased prevalence in IBD mucosa augment in vitro utilization of mucin by other bacteria. *The American journal of gastroenterology* **2010**.
100. Ahn, J.; Sinha, R.; Pei, Z.; Dominianni, C.; Wu, J.; Shi, J.; Goedert, J.J.; Hayes, R.B.; Yang, L. Human Gut Microbiome and Risk for Colorectal Cancer. *Journal of the National Cancer Institute* **2013**, *105*, 1907-1911.
101. Watanabe, T.; Tada, M.; Nagai, H.; Sasaki, S.; Nakao, M. < i> Helicobacter pylori</i> infection induces gastric cancer in Mongolian gerbils. *Gastroenterology* **1998**, *115*, 642-648.
102. Osaki, T.; Matsuki, T.; Asahara, T.; Zaman, C.; Hanawa, T.; Yonezawa, H.; Kurata, S.; Woo, T.D.-h.; Nomoto, K.; Kamiya, S. Comparative analysis of gastric bacterial microbiota in Mongolian gerbils after long-term infection with< i> Helicobacter pylori</i>. *Microbial pathogenesis* **2012**, *53*, 12-18.
103. Zaman, C.; Osaki, T.; Hanawa, T.; Yonezawa, H.; Kurata, S.; Kamiya, S. Analysis of the microbial ecology between Helicobacter pylori and the gastric microbiota of Mongolian gerbils. *Journal of medical microbiology* **2014**, *63*, 129-137.
104. Aviles-Jimenez, F.; Vazquez-Jimenez, F.; Medrano-Guzman, R.; Mantilla, A.; Torres, J. Stomach microbiota composition varies between patients with non-atrophic gastritis and patients with intestinal type of gastric cancer. *Scientific reports* **2014**, *4*.
105. Dicksved, J.; Lindberg, M.; Rosenquist, M.; Enroth, H.; Jansson, J.K.; Engstrand, L. Molecular characterization of the stomach microbiota in patients with gastric cancer and in controls. *Journal of medical microbiology* **2009**, *58*, 509-516.
106. Shin, C.M.; Kim, N.; Lee, H.S.; Park, J.H.; Ahn, S.; Kang, G.H.; Kim, J.M.; Kim, J.S.; Lee, D.H.; Jung, H.C. Changes in aberrant DNA methylation after Helicobacter pylori eradication: A long−term follow−up study. *International Journal of Cancer* **2013**, *133*, 2034-2042.
107. Cai, X.; Carlson, J.; Stoicov, C.; Li, H.; Wang, T.C.; Houghton, J. < i> Helicobacter felis</i> Eradication Restores Normal Architecture and Inhibits Gastric Cancer Progression in C57BL/6 Mice. *Gastroenterology* **2005**, *128*, 1937-1952.
108. Wang, T.C.; Goldenring, J.R.; Dangler, C.; Ito, S.; Mueller, A.; Jeon, W.K.; Koh, T.J.; Fox, J.G. Mice lacking secretory phospholipase A< sub> 2</sub> show altered apoptosis and differentiation with< i> Helicobacter felis</i> infection. *Gastroenterology* **1998**, *114*, 675-689.
109. Moen, E.L.; Wen, S.; Anwar, T.; Cross-Knorr, S.; Brilliant, K.; Birnbaum, F.; Rahaman, S.; Sedivy, J.M.; Moss, S.F.; Chatterjee, D. Regulation of RKIP function by Helicobacter pylori in gastric cancer. *PloS one* **2012**, *7*, e37819.
110. Yang, L.; Chaudhary, N.; Baghdadi, J.; Pei, Z. Microbiome in Reflux Disorders and Esophageal Adenocarcinoma. *The Cancer Journal* **2014**, *20*, 207-210.
111. Anderson, L.A.; Murphy, S.J.; Johnston, B.T.; Watson, R.; Ferguson, H.; Bamford, K.B.; Ghazy, A.; McCarron, P.; McGuigan, J.; Reynolds, J.V. Relationship between

Helicobacter pylori infection and gastric atrophy and the stages of the oesophageal inflammation, metaplasia, adenocarcinoma sequence: results from the FINBAR case–control study. *Gut* **2008**, *57*, 734-739.
112. Yang, L.; Lu, X.; Nossa, C.W.; Francois, F.; Peek, R.M.; Pei, Z. Inflammation and intestinal metaplasia of the distal esophagus are associated with alterations in the microbiome. *Gastroenterology* **2009**, *137*, 588-597.
113. Narikiyo, M.; Tanabe, C.; Yamada, Y.; Igaki, H.; Tachimori, Y.; Kato, H.; Muto, M.; Montesano, R.; Sakamoto, H.; Nakajima, Y. Frequent and preferential infection of Treponema denticola, Streptococcus mitis, and Streptococcus anginosus in esophageal cancers. *Cancer science* **2004**, *95*, 569-574.
114. Farrell, J.J.; Zhang, L.; Zhou, H.; Chia, D.; Elashoff, D.; Akin, D.; Paster, B.J.; Joshipura, K.; Wong, D.T. Variations of oral microbiota are associated with pancreatic diseases including pancreatic cancer. *Gut* **2012**, *61*, 582-588.
115. Gong, H.-L.; Shi, Y.; Zhou, L.; Wu, C.-P.; Cao, P.-Y.; Tao, L.; Xu, C.; Hou, D.-S.; Wang, Y.-Z. The composition of microbiome in larynx and the throat biodiversity between laryngeal squamous cell carcinoma patients and control population. *PloS one* **2013**, *8*, e66476.
116. Sharma, V.; Chauhan, V.S.; Nath, G.; Kumar, A.; Shukla, V.K. Role of bile bacteria in gallbladder carcinoma. *Hepato-gastroenterology* **2007**, *54*, 1622.
117. Shukla, V.; Tiwari, S.; Roy, S. Biliary bile acids in cholelithiasis and carcinoma of the gall bladder. *European journal of cancer prevention* **1993**, *2*, 155-160.
118. Walboomers, J.M.; Jacobs, M.V.; Manos, M.M.; Bosch, F.X.; Kummer, J.A.; Shah, K.V.; Snijders, P.J.; Peto, J.; Meijer, C.J.; Munoz, N. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *The Journal of pathology* **1999**, *189*, 12-19.
119. Mitrani-Rosenbaum, S.; Tsvieli, R.; Tur-Kaspa, R. Oestrogen stimulates differential transcription of human papillomavirus type 16 in SiHa cervical carcinoma cells. *Journal of general virology* **1989**, *70*, 2227-2232.
120. Riley, R.R.; Duensing, S.; Brake, T.; Münger, K.; Lambert, P.F.; Arbeit, J.M. Dissection of human papillomavirus E6 and E7 function in transgenic mouse models of cervical carcinogenesis. *Cancer research* **2003**, *63*, 4862-4871.
121. Chung, S.-H.; Wiedmeyer, K.; Shai, A.; Korach, K.S.; Lambert, P.F. Requirement for estrogen receptor α in a mouse model for human papillomavirus–associated cervical cancer. *Cancer research* **2008**, *68*, 9928-9934.
122. Elson, D.A.; Riley, R.R.; Lacey, A.; Thordarson, G.; Talamantes, F.J.; Arbeit, J.M. Sensitivity of the cervical transformation zone to estrogen-induced squamous carcinogenesis. *Cancer research* **2000**, *60*, 1267-1275.
123. Lombardi, P.; Goldin, B.; Boutin, E.; Gorbach, S.L. Metabolism of androgens and estrogens by human fecal microorganisms. *Journal of steroid biochemistry* **1978**, *9*, 795-801.
124. D'Souza, G.; Kreimer, A.R.; Viscidi, R.; Pawlita, M.; Fakhry, C.; Koch, W.M.; Westra, W.H.; Gillison, M.L. Case–control study of human papillomavirus and oropharyngeal cancer. *New England Journal of Medicine* **2007**, *356*, 1944-1956.
125. Coussens, L.M.; Werb, Z. Inflammation and cancer. *Nature* **2002**, *420*, 860-867.
126. Nugent, J.L.; McCoy, A.N.; Addamo, C.J.; Jia, W.; Sandler, R.S.; Keku, T.O. Altered Tissue Metabolites Correlate with Microbial Dysbiosis in Colorectal Adenomas. *J. Proteome Res.* **2014**, *13*, 1921-1929.

127. Forsythe, P.; Bienenstock, J. Immunomodulation by commensal and probiotic bacteria. *Immunological investigations* **2010**, *39*, 429-448.
128. Chang, P.V.; Hao, L.; Offermanns, S.; Medzhitov, R. The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition. *Proceedings of the National Academy of Sciences* **2014**, 201322269.
129. Akare, S.; Jean−Louis, S.; Chen, W.; Wood, D.J.; Powell, A.A.; Martinez, J.D. Ursodeoxycholic acid modulates histone acetylation and induces differentiation and senescence. *International journal of cancer* **2006**, *119*, 2958-2969.
130. Miao, X.P.; Ouyang, Q.; Li, H.Y.; Zhao, Z.Q.; Pan, Y.; Wang, Z.W. Ursodeoxycholic acid for the prevention of colorectal adenomas and carcinomas. *The Cochrane Library* **2013**.
131. Eaton, J.E.; Silveira, M.G.; Pardi, D.S.; Sinakos, E.; Kowdley, K.V.; Luketic, V.A.; Harrison, M.E.; McCashland, T.; Befeler, A.S.; Harnois, D. High-dose ursodeoxycholic acid is associated with the development of colorectal neoplasia in patients with ulcerative colitis and primary sclerosing cholangitis. *The American journal of gastroenterology* **2011**, *106*, 1638-1645.
132. Ajouz, H.; Mukherji, D.; Shamseddine, A. Secondary bile acids: an underrecognized cause of colon cancer. *World Journal of Surgical Oncology* **2014**, *12*, 164.
133. Yoshimoto, S.; Loo, T.M.; Atarashi, K.; Kanda, H.; Sato, S.; Oyadomari, S.; Iwakura, Y.; Oshima, K.; Morita, H.; Hattori, M. Obesity-induced gut microbial metabolite promotes liver cancer through senescence secretome. *Nature* **2013**, *499*, 97-101.
134. Wang, H.; Chen, J.; Hollister, K.; Sowers, L.C.; Forman, B.M. Endogenous bile acids are ligands for the nuclear receptor FXR/BAR. *Molecular cell* **1999**, *3*, 543-553.
135. Deuschle, U.; Schüler, J.; Schulz, A.; Schlüter, T.; Kinzel, O.; Abel, U.; Kremoser, C. FXR controls the tumor suppressor NDRG2 and FXR agonists reduce liver tumor growth and metastasis in an orthotopic mouse xenograft model. *PloS one* **2012**, *7*, e43044.
136. Inagaki, T.; Moschetta, A.; Lee, Y.-K.; Peng, L.; Zhao, G.; Downes, M.; Ruth, T.Y.; Shelton, J.M.; Richardson, J.A.; Repa, J.J. Regulation of antibacterial defense in the small intestine by the nuclear bile acid receptor. *Proceedings of the National Academy of Sciences of the United States of America* **2006**, *103*, 3920-3925.
137. Adlercreutz, H.; Martin, F.; Pulkkinen, M.; Dencker, H.; Rimer, U.; Sjoberg, N.-O.; Tikkanen, M. Intestinal Metabolism of Estrogens 1. *The Journal of Clinical Endocrinology & Metabolism* **1976**, *43*, 497-505.
138. Woolcott, C.G.; Shvetsov, Y.B.; Stanczyk, F.Z.; Wilkens, L.R.; White, K.K.; Caberto, C.; Henderson, B.E.; Le Marchand, L.; Kolonel, L.N.; Goodman, M.T. Plasma sex hormone concentrations and breast cancer risk in an ethnically diverse population of postmenopausal women: the Multiethnic Cohort Study. *Endocrine-related cancer* **2010**, *17*, 125-134.
139. Mackenzie, I. The production of mammary cancer in rats using oestrogens. *Br. J. Cancer* **1955**, *9*, 284.
140. Hill, M.; Goddard, P.; Williams, R. Gut bacteria and aetiology of cancer of the breast. *The Lancet* **1971**, *298*, 472-473.
141. Muti, P.; Bradlow, H.L.; Micheli, A.; Krogh, V.; Freudenheim, J.L.; Schünemann, H.J.; Stanulla, M.; Yang, J.; Sepkovic, D.W.; Trevisan, M. Estrogen metabolism and risk of breast cancer: a prospective study of the 2: 16α-hydroxyestrone ratio in premenopausal and postmenopausal women. *Epidemiology* **2000**, *11*, 635-640.

142. Ou, J.; Carbonero, F.; Zoetendal, E.G.; DeLany, J.P.; Wang, M.; Newton, K.; Gaskins, H.R.; O'Keefe, S.J. Diet, microbiota, and microbial metabolites in colon cancer risk in rural Africans and African Americans. *The American journal of clinical nutrition* **2013**, *98*, 111-120.
143. Xie, G.; Zhang, S.; Zheng, X.; Jia, W. Metabolomics approaches for characterizing metabolic interactions between host and its commensal microbes. *Electrophoresis* **2013**, *34*, 2787-2798.
144. Stecher, B.; Maier, L.; Hardt, W.-D. 'Blooming'in the gut: how dysbiosis might contribute to pathogen evolution. *Nature Reviews Microbiology* **2013**, *11*, 277-284.
145. Keszei, A.P.; Goldbohm, R.A.; Schouten, L.J.; Jakszyn, P.; van den Brandt, P.A. Dietary N-nitroso compounds, endogenous nitrosation, and the risk of esophageal and gastric cancer subtypes in the Netherlands Cohort Study. *The American journal of clinical nutrition* **2013**, *97*, 135-146.
146. Giacosa, A.; Barale, R.; Bavaresco, L.; Gatenby, P.; Gerbi, V.; Janssens, J.; Johnston, B.; Kas, K.; La Vecchia, C.; Mainguet, P. Cancer prevention in Europe: the Mediterranean diet as a protective choice. *European Journal of Cancer Prevention* **2013**, *22*, 90-95.
147. Vipperla, K.; Ou, J.; Wahl, E.; Ruder, E.; O'Keefe, S. A 14-day in-house dietary modification of a 'Western'diet to an 'African'diet changes the microbiota, its metabolome, and biomarkers of colon cancer risk (825.5). *The FASEB Journal* **2014**, *28*, 825.825.
148. Song, Y.; Garg, S.; Girotra, M.; Maddox, C.; von Rosenvinge, E.C.; Dutta, A.; Dutta, S.; Fricke, W.F. Microbiota Dynamics in Patients Treated with Fecal Microbiota Transplantation for Recurrent Clostridium difficile Infection. *PloS one* **2013**, *8*, e81330.
149. Marzotto, M.; Maffeis, C.; Paternoster, T.; Ferrario, R.; Rizzotti, L.; Pellegrino, M.; Dellaglio, F.; Torriani, S. < i> Lactobacillus paracasei</i> A survives gastrointestinal passage and affects the fecal microbiota of healthy infants. *Research in microbiology* **2006**, *157*, 857-866.
150. Matsumoto, M.; Aranami, A.; Ishige, A.; Watanabe, K.; Benno, Y. LKM512 yogurt consumption improves the intestinal environment and induces the T−helper type 1 cytokine in adult patients with intractable atopic dermatitis. *Clinical & Experimental Allergy* **2007**, *37*, 358-370.
151. Clarke, S.F.; Murphy, E.F.; O'Sullivan, O.; Lucey, A.J.; Humphreys, M.; Hogan, A.; Hayes, P.; O'Reilly, M.; Jeffery, I.B.; Wood-Martin, R.*, et al.* Exercise and associated dietary extremes impact on gut microbial diversity. *Gut* **2014**, 10.1136/gutjnl-2013-306541.
152. Turnbaugh, P.J.; Ley, R.E.; Mahowald, M.A.; Magrini, V.; Mardis, E.R.; Gordon, J.I. An obesity-associated gut microbiome with increased capacity for energy harvest. *nature* **2006**, *444*, 1027-1131.
153. Neish, A.S. Microbes in Gastrointestinal Health and Disease. *Gastroenterology* **2009**, *136*, 65-80, http://dx.doi.org/10.1053/j.gastro.2008.10.080.
154. Kelly, D.; Conway, S.; Aminov, R. Commensal gut bacteria: mechanisms of immune modulation. *Trends in Immunology* **2005**, *26*, 326-333, http://dx.doi.org/10.1016/j.it.2005.04.008.
155. Selgrad, M.; Malfertheiner, P.; Fini, L.; Goel, A.; Boland, C.R.; Ricciardiello, L. The role of viral and bacterial pathogens in gastrointestinal cancer. *Journal of Cellular Physiology* **2008**, *216*, 378-388, 10.1002/jcp.21427.

156. Klein, R.S.; Catalano, M.T.; Edberg, S.C.; Casey, J.I.; Steigbigel, N.H.; . Streptococcus bovis septicemia and carcinoma of the colon. *Ann. Intern. Med.* **1979**, *91*, 560-562.
157. Leport, C.; Bure, J.; Leport, J.; Vilde, J.L. Incidence of colonic lesions in Streptococcus bovis and enterococcal endocarditis. . *Lancet* **1987**, *1*, 748–749.
158. Hill, M.J.; Drasar, B.S.; Aries, V.; Crowther, J.S.; Hawksworth, G.; Williams, R.E.O. BACTERIA AND ÆTIOLOGY OF CANCER OF LARGE BOWEL. *The Lancet* **1971**, *297*, 95-100, http://dx.doi.org/10.1016/S0140-6736(71)90837-3.
159. Maclennan, R.; Jensen, O.M. Dietary fibre, transit-time, faecal bacteria, steroids, and colon cancer in two Scandinavian populations. Report from the International Agency for Research on Cancer Intestinal Microecology Group. *Lancet* **1977**, *2*, 207-211.
160. Moore, W.E.; Moore, L.H. Intestinal floras of populations that have a high risk of colon cancer. *Applied and Environmental Microbiology* **1995**, *61*, 3202-3207.
161. Castellarin, M.; Warren, R.L.; Freeman, J.D.; Dreolini, L.; Krzywinski, M.; Strauss, J.; Barnes, R.; Watson, P.; Allen-Vercoe, E.; Moore, R.A.*, et al.* Fusobacterium nucleatum infection is prevalent in human colorectal carcinoma. *Genome Research* **2011**, 10.1101/gr.126516.111.
162. Kostic, A.D.; Gevers, D.; Pedamallu, C.S.; Michaud, M.; Duke, F.; Earl, A.M.; Ojesina, A.I.; Jung, J.; Bass, A.J.; Tabernero, J.*, et al.* Genomic analysis identifies association of Fusobacterium with colorectal carcinoma. *Genome Research* **2011**, 10.1101/gr.126573.111.
163. Chen, W.; Liu, F.; Ling, Z.; Tong, X.; Xiang, C. Human intestinal lumen and mucosa-associated microbiota in patients with colorectal cancer. *PloS one* **2012**, *7*, e39743.
164. Marchesi, J.R.; Dutilh, B.E.; Hall, N.; Peters, W.H.M.; Roelofs, R.; Boleij, A.; Tjalsma, H. Towards the Human Colorectal Cancer Microbiome. *PLoS ONE* **2011**, *6*, e20447, 10.1371/journal.pone.0020447.
165. Huycke, M.M.; Gaskins, H.R. Commensal Bacteria, Redox Stress, and Colorectal Cancer: Mechanisms and Models. *Experimental Biology and Medicine* **2004**, *229*, 586-597.
166. Nicholson, J.K.; Lindon, J.C. Systems biology: Metabonomics. *Nature* **2008**, *455*, 1054-1056.
167. Nicholls, A.W.; Mortishire-Smith, R.J.; Nicholson, J.K. NMR Spectroscopic-Based Metabonomic Studies of Urinary Metabolite Variation in Acclimatizing Germ-Free Rats. *Chemical Research in Toxicology* **2003**, *16*, 1395-1404, 10.1021/tx0340293.
168. Martin, F.-P.J.; Sprenger, N.; Montoliu, I.; Rezzi, S.; Kochhar, S.; Nicholson, J.K. Dietary Modulation of Gut Functional Ecology Studied by Fecal Metabonomics. *Journal of Proteome Research* **2010**, *9*, 5284-5295, 10.1021/pr100554m.
169. Kinross, J.; Darzi, A.; Nicholson, J. Gut microbiome-host interactions in health and disease. *Genome Medicine* **2011**, *3*, 14.
170. Jacobs, D.M.; Deltimple, N.; van Velzen, E.; van Dorsten, F.A.; Bingham, M.; Vaughan, E.E.; van Duynhoven, J. 1H NMR metabolite profiling of feces as a tool to assess the impact of nutrition on the human microbiome. *NMR in Biomedicine* **2008**, *21*, 615-626, 10.1002/nbm.1233.
171. Fierer, N.; Hamady, M.; Lauber, C.L.; Knight, R. The influence of sex, handedness, and washing on the diversity of hand surface bacteria. *Proceedings of the National Academy of Sciences* **2008**, *105*, 17994-17999, 10.1073/pnas.0807920105.

172. Schloss, P.D.; Westcott, S.L.; Ryabin, T.; Hall, J.R.; Hartmann, M.; Hollister, E.B.; Lesniewski, R.A.; Oakley, B.B.; Parks, D.H.; Robinson, C.J.*, et al.* Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities. *Applied and Environmental Microbiology* **2009**, *75*, 7537-7541, 10.1128/aem.01541-09.
173. Wang, Q.; Garrity, G.M.; Tiedje, J.M.; Cole, J.R. Naïve Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy. *Applied and Environmental Microbiology* **2007**, *73*, 5261-5267, 10.1128/aem.00062-07.
174. Huse, S.M.; Welch, D.M.; Morrison, H.G.; Sogin, M.L. Ironing out the wrinkles in the rare biosphere through improved OTU clustering. *Environmental Microbiology* **2010**, *12*, 1889-1898, 10.1111/j.1462-2920.2010.02193.x.
175. Smith, C.A.; Want, E.J.; O'Maille, G.; Abagyan, R.; Siuzdak, G. XCMS: Processing Mass Spectrometry Data for Metabolite Profiling Using Nonlinear Peak Alignment, Matching, and Identification. *Analytical Chemistry* **2006**, *78*, 779-787, 10.1021/ac051437y.
176. Stein, S.E. An integrated method for spectrum extraction and compound identification from gas chromatography/mass spectrometry data. *J Am Soc Mass Spectrom* **1999**, *10*, 770-781, 10.1016/S1044-0305(99)00047-1.
177. Andersson, A.F.; Lindberg, M.; Jakobsson, H.; Bäckhed, F.; Nyrén, P.; Engstrand, L. Comparative Analysis of Human Gut Microbiota by Barcoded Pyrosequencing. *PLoS ONE* **2008**, *3*, e2836.
178. Yue, J.C.; Clayton, M.K. A Similarity Measure Based on Species Proportions. *Communications in Statistics - Theory and Methods* **2005**, *34*, 2123-2131, 10.1080/STA-200066418.
179. De Filippo, C.; Cavalieri, D.; Di Paola, M.; Ramazzotti, M.; Poullet, J.B.; Massart, S.; Collini, S.; Pieraccini, G.; Lionetti, P. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proceedings of the National Academy of Sciences* **2010**, *107*, 14691-14696, 10.1073/pnas.1005963107.
180. Shen, X.J.; Rawls, J.F.; Randall, T.A.; Burcall, L.; Mpande, C.; Jenkins, N.; Jovov, B.; Abdo, Z.; Sandler, R.S.; Keku, T.O. Molecular characterization of mucosal adherent bacteria and associations with colorectal adenomas. *Gut microbes* **2010**, *1*, 138-147.
181. Joossens, M.; Huys, G.; Cnockaert, M.; De Preter, V.; Verbeke, K.; Rutgeerts, P.; Vandamme, P.; Vermeire, S. Dysbiosis of the faecal microbiota in patients with Crohn's disease and their unaffected relatives. *Gut* **2011**, 10.1136/gut.2010.223263.
182. Png, C.W.; Linden, S.K.; Gilshenan, K.S.; Zoetendal, E.G.; McSweeney, C.S.; Sly, L.I.; McGuckin, M.A.; Florin, T.H.J. Mucolytic Bacteria With Increased Prevalence in IBD Mucosa Augment In Vitro Utilization of Mucin by Other Bacteria. *Am J Gastroenterol* **2010**, http://www.nature.com/ajg/journal/vaop/ncurrent/suppinfo/ajg2010281s1.html.
183. Zella, G.C.; Hait, E.J.; Glavan, T.; Gevers, D.; Ward, D.V.; Kitts, C.L.; Korzenik, J.R. Distinct microbiome in pouchitis compared to healthy pouches in ulcerative colitis and familial adenomatous polyposis. *Inflammatory Bowel Diseases* **2011**, *17*, 1092-1100, 10.1002/ibd.21460.
184. Byrd, J.C.; Bresalier, R.S. Mucins and mucin binding proteins in colorectal cancer: Colorectal Cancer. *Cancer and Metastasis Reviews* **2004**, *23*, 77-99, 10.1023/A:1025815113599.

185. Deloménie, C.; Fouix, S.; Longuemaux, S.; Brahimi, N.m.; Bizet, C.; Picard, B.; Denamur, E.; Dupret, J.-M. Identification and Functional Characterization of Arylamine N-Acetyltransferases in Eubacteria: Evidence for Highly Selective Acetylation of 5-Aminosalicylic Acid. *Journal of Bacteriology* **2001**, *183*, 3417-3427, 10.1128/jb.183.11.3417-3427.2001.
186. Bartosch, S.; Fite, A.; Macfarlane, G.T.; McMurdo, M.E.T. Characterization of Bacterial Communities in Feces from Healthy Elderly Volunteers and Hospitalized Elderly Patients by Using Real-Time PCR and Effects of Antibiotic Treatment on the Fecal Microbiota. *Applied and Environmental Microbiology* **2004**, *70*, 3575-3581, 10.1128/aem.70.6.3575-3581.2004.
187. Sartor, R.B. Therapeutic correction of bacterial dysbiosis discovered by molecular techniques. *Proceedings of the National Academy of Sciences* **2008**, *105*, 16413-16414, 10.1073/pnas.0809363105.
188. Williams, E.A.; Coxhead, J.M.; Mathers, J.C. Anti-cancer effects of butyrate: use of micro-array technology to investigate mechanisms. *Proceedings of the Nutrition Society* **2003**, *62*, 107-115, doi:10.1079/PNS2002230.
189. Matthews, G.M.; Howarth, G.S.; Butler, R.N. Short-Chain Fatty Acids Induce Apoptosis in Colon Cancer Cells Associated with Changes to Intracellular Redox State and Glucose Metabolism. *Chemotherapy* **2012**, *58*, 102-109.
190. Weaver, G.A.; Krause, J.A.; Miller, T.L.; Wolin, M.J. Short chain fatty acid distributions of enema samples from a sigmoidoscopy population: an association of high acetate and low butyrate ratios with adenomatous polyps and colon cancer. *Gut* **1988**, *29*, 1539-1543, 10.1136/gut.29.11.1539.
191. Sato, K.; Tsuchihara, K.; Fujii, S.; Sugiyama, M.; Goya, T.; Atomi, Y.; Ueno, T.; Ochiai, A.; Esumi, H. Autophagy Is Activated in Colorectal Cancer Cells and Contributes to the Tolerance to Nutrient Deprivation. *Cancer Research* **2007**, *67*, 9677-9684, 10.1158/0008-5472.can-07-1462.
192. Hirayama, A.; Kami, K.; Sugimoto, M.; Sugawara, M.; Toki, N.; Onozuka, H.; Kinoshita, T.; Saito, N.; Ochiai, A.; Tomita, M.*, et al.* Quantitative Metabolome Profiling of Colon and Stomach Cancer Microenvironment by Capillary Electrophoresis Time-of-Flight Mass Spectrometry. *Cancer Research* **2009**, *69*, 4918-4925, 10.1158/0008-5472.can-08-4806.
193. Monleón, D.; Morales, J.M.; Barrasa, A.; López, J.A.; Vázquez, C.; Celda, B. Metabolite profiling of fecal water extracts from human colorectal cancer. *NMR in Biomedicine* **2009**, *22*, 342-348, 10.1002/nbm.1345.
194. Byrd, J.; Bresalier, R. Mucins and mucin binding proteins in colorectal cancer. *Cancer and Metastasis Reviews* **2004**, *23*, 77-99, 10.1023/a:1025815113599.
195. Akare, S.; Jean-Louis, S.; Chen, W.; Wood, D.J.; Powell, A.A.; Martinez, J.D. Ursodeoxycholic acid modulates histone acetylation and induces differentiation and senescence. *International Journal of Cancer* **2006**, *119*, 2958-2969, 10.1002/ijc.22231.
196. Lepercq, P.; Gérard, P.; Béguet, F.; Grill, J.-P.; Relano, P.; Cayuela, C.; Juste, C. Isolates from Normal Human Intestinal Flora but not Lactic Acid Bacteria Exhibit 7a- and b-Hydroxysteroid Dehydrogenase Activities. *Microbial Ecology in Health and Disease* **2011**, *16*, 1651-2235.
197. Tjalsma, H.; Boleij, A.; Marchesi, J.R.; Dutilh, B.E. A bacterial driver–passenger model for colorectal cancer: beyond the usual suspects. *Nat Rev Micro* **2012**, *10*, 575-582.

198. Cicero, A.; Derosa, G. Rice bran and its main components: Potential role in the management of coronary risk factors. *Curr. Top. Nutraceutical Res.* **2005**, *3*, 29-46.
199. Cheng, H.-H.; Huang, H.-Y.; Chen, Y.-Y.; Huang, C.-L.; Chang, C.-J.; Chen, H.-L.; Lai, M.-H. Ameliorative effects of stabilized rice bran on type 2 diabetes patients. *Ann. Nutr. Metabol.* **2009**, *56*, 45-51.
200. De Munter, J.S.; Hu, F.B.; Spiegelman, D.; Franz, M.; van Dam, R.M. Whole grain, bran, and germ intake and risk of type 2 diabetes: a prospective cohort study and systematic review. *PLoS Med.* **2007**, *4*, e261, doi:10.1371/journal.pmed.0040261.
201. Jariwalla, R. Rice-bran products: phytonutrients with potential applications in preventive and clinical medicine. *Drugs Exp. Clin. Res.* **2000**, *27*, 17-26.
202. Kim, T.H.; Kim, E.K.; Lee, M.-S.; Lee, H.-K.; Hwang, W.S.; Choe, S.J.; Kim, T.-Y.; Han, S.J.; Kim, H.J.; Kim, D.J. Intake of brown rice lees reduces waist circumference and improves metabolic parameters in type 2 diabetes. *Nutr. Res.* **2011**, *31*, 131-138.
203. Phutthaphadoong, S.; Yamada, Y.; Hirata, A.; Tomita, H.; Hara, A.; Limtrakul, P.; Iwasaki, T.; Kobayashi, H.; Mori, H. Chemopreventive effect of fermented brown rice and rice bran (FBRA) on the inflammation-related colorectal carcinogenesis in ApcMin/+ mice. *Oncol. Rep.* **2010**, *23*, 53-59.
204. Verschoyle, R.; Greaves, P.; Cai, H.; Edwards, R.; Steward, W.; Gescher, A. Evaluation of the cancer chemopreventive efficacy of rice bran in genetic mouse models of breast, prostate and intestinal carcinogenesis. *Br. J. Cancer* **2007**, *96*, 248-254.
205. Komiyama, Y.; Andoh, A.; Fujiwara, D.; Ohmae, H.; Araki, Y.; Fujiyama, Y.; Mitsuyama, K.; Kanauchi, O. New prebiotics from rice bran ameliorate inflammation in murine colitis models through the modulation of intestinal homeostasis and the mucosal immune system. *Scand. J. Gastroenterol.* **2011**, *46*, 40-52.
206. Flint, H.J. The impact of nutrition on the human microbiome. *Nutr. Rev.* **2012**, *70*, S10-S13.
207. Kovatcheva-Datchary, P.; Arora, T. Nutrition, the gut microbiome and the metabolic syndrome. *Best Pract. Res. Clin. Gastroenterol.* **2013**, *27*, 59-72.
208. Nicholson, J.K.; Holmes, E.; Kinross, J.; Burcelin, R.; Gibson, G.; Jia, W.; Pettersson, S. Host-gut microbiota metabolic interactions. *Science* **2012**, *336*, 1262-1267.
209. Suzuki, T.; Yoshida, S.; Hara, H. Physiological concentrations of short-chain fatty acids immediately suppress colonic epithelial permeability. *Br. J. Nutr.* **2008**, *100*, 297-305.
210. Chang, P.V.; Hao, L.; Offermanns, S.; Medzhitov, R. The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition. *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 2247–2252.
211. Maslowski, K.M.; Vieira, A.T.; Ng, A.; Kranich, J.; Sierro, F.; Yu, D.; Schilter, H.C.; Rolph, M.S.; Mackay, F.; Artis, D. Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. *Nature* **2009**, *461*, 1282-1286.
212. Vipperla, K.; O'Keefe, S.J. The microbiota and its metabolites in colonic mucosal health and cancer risk. *Nutr. Clin. Pract.* **2012**, *27*, 624-635.
213. Waldecker, M.; Kautenburger, T.; Daumann, H.; Busch, C.; Schrenk, D. Inhibition of histone-deacetylase activity by short-chain fatty acids and some polyphenol metabolites formed in the colon. *J. Nutr. Biochem.* **2008**, *19*, 587-593.
214. Schloss, P.D.; Westcott, S.L.; Ryabin, T.; Hall, J.R.; Hartmann, M.; Hollister, E.B.; Lesniewski, R.A.; Oakley, B.B.; Parks, D.H.; Robinson, C.J*., et al.* Introducing mothur:

open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* **2009**, *75*, 7537-7541.
215. Pruesse, E.; Quast, C.; Knittel, K.; Fuchs, B.M.; Ludwig, W.; Peplies, J.; Glockner, F.O. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic acids research* **2007**, *35*, 7188-7196, 10.1093/nar/gkm864.
216. Wang, Q.; Garrity, G.M.; Tiedje, J.M.; Cole, J.R. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* **2007**, *73*, 5261-5267.
217. Sheneman, L.; Evans, J.; Foster, J.A. Clearcut: a fast implementation of relaxed neighbor joining. *Bioinformatics* **2006**, *22*, 2823-2824.
218. Smith, C.A.; Want, E.J.; O'Maille, G.; Abagyan, R.; Siuzdak, G. XCMS: processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification. *Anal. Chem.* **2006**, *78*, 779-787.
219. Davies, T. The new automated mass spectrometry deconvolution and identification system (AMDIS). *Spectrosc. Eur.* **1998**, *10*, 24-27.
220. The R Core Team. R: A language and environment for statistical computing. Available online: http://web.mit.edu/r_v3.0.1/fullrefman.pdf (accessed on 25 August 2013)
221. White, J.R.; Nagarajan, N.; Pop, M. Statistical Methods for Detecting Differentially Abundant Features in Clinical Metagenomic Samples. *PLoS Comp. Biol.* **2009**, *5*, e1000352, 10.1371/journal.pcbi.1000352.
222. Storey, J.D.; Tibshirani, R. Statistical significance for genomewide studies. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 9440-9445.
223. U. S. Department of Agriculture, A.R.S. USDA National Nutrient Database for Standard Reference, Release 26. Nutrient Data Laboratory Home Page, http://www.ars.usda.gov/ba/bhnrc/ndl (accessed on 5 February 2013).
224. Topping, D.L.; Clifton, P.M. Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. *Physiol. Rev.* **2001**, *81*, 1031-1064.
225. Vernocchi, P.; Vannini, L.; Gottardi, D.; Del Chierico, F.; Serrazanetti, D.I.; Ndagijimana, M.; Guerzoni, M.E. Integration of datasets from different analytical techniques to assess the impact of nutrition on human metabolome. *Front. Cell. Infect. Microbiol.* **2012**, *2*, 156, doi:10.3389/fcimb.2012.00156.
226. Wishart, D.S.; Jewison, T.; Guo, A.C.; Wilson, M.; Knox, C.; Liu, Y.; Djoumbou, Y.; Mandal, R.; Aziat, F.; Dong, E. HMDB 3.0—the human metabolome database in 2013. *Nucleic Acids Res.* **2013**, *41*, D801-D807.
227. Forster, G.M.; Raina, K.; Kumar, A.; Kumar, S.; Agarwal, R.; Chen, M.-H.; Bauer, J.E.; McClung, A.M.; Ryan, E.P. Rice varietal differences in bioactive bran components for inhibition of colorectal cancer cell growth. *Food Chem.* **2013**, *141*, 1545-1552.
228. Nemoto, H.; Ikata, K.; Arimochi, H.; Iwasaki, T.; Ohnishi, Y.; Kuwahara, T.; Kataoka, K. Effects of fermented brown rice on the intestinal environments in healthy adult. *J. Investig. Med.* **2011**, *58*, 235-245.
229. Yang, J.; Keshavarzian, A.; Rose, D.J. Impact of dietary fiber fermentation from cereal grains on metabolite production by the fecal microbiota from normal weight and obese individuals. *J. Med. Food* **2013**, *16*, 862-867.

230. Blachier, F.; Mariotti, F.; Huneau, J.; Tome, D. Effects of amino acid-derived luminal metabolites on the colonic epithelium and physiopathological consequences. *Amino Acids* **2007**, *33*, 547-562.
231. Le Gall, G.; Noor, S.O.; Ridgway, K.; Scovell, L.; Jamieson, C.; Johnson, I.T.; Colquhoun, I.J.; Kemsley, E.K.; Narbad, A. Metabolomics of fecal extracts detects altered metabolic activity of gut microbiota in ulcerative colitis and irritable bowel syndrome. *J. Proteome Res.* **2011**, *10*, 4208-4218.
232. Boudry, G.; Jamin, A.; Chatelais, L.; Gras-Le Guen, C.; Michel, C.; Le Huërou-Luron, I. Dietary protein excess during neonatal life alters colonic Microbiota and mucosal response to inflammatory mediators later in life in female pigs. *J. Nutr.* **2013**, *143*, 1225-1232.
233. Ran-Ressler, R.R.; Khailova, L.; Arganbright, K.M.; Adkins-Rieck, C.K.; Jouni, Z.E.; Koren, O.; Ley, R.E.; Brenna, J.T.; Dvorak, B. Branched chain fatty acids reduce the incidence of necrotizing enterocolitis and alter gastrointestinal microbial ecology in a neonatal rat model. *PloS One* **2011**, *6*, e29032, doi:10.1371/journal.pone.0029032.
234. Hu, G.; Yu, W. Binding of cholesterol and bile acid to hemicelluloses from rice bran. *Int. J. Food Sci. Nutr.* **2013**, *64*, 461-466.
235. Gestel, G.; Besancon, P.; Rouanet, J.-M. Comparative evaluation of the effects of two different forms of dietary fibre (rice bran vs. wheat bran) on rat colonic mucosa and faecal microflora. *Ann. Nutr. Metabol.* **1994**, *38*, 249-256.
236. Robson, J. Lipid modification: cardiovascular risk assessment and the modification of blood lipids for the primary and secondary prevention of cardiovascular disease. *Heart* **2008**, *94*, 1331-1332, 10.1136/hrt.2008.150979.
237. Rukmini, C.; Raghuram, T.C. Nutritional and biochemical aspects of the hypolipidemic action of rice bran oil: a review. *J. Am. Coll. Nutr.* **1991**, *10*, 593-601.
238. Akihisa, T.; Yasukawa, K.; Yamaura, M.; Ukiya, M.; Kimura, Y.; Shimizu, N.; Arai, K. Triterpene alcohol and sterol ferulates from rice bran and their anti-inflammatory effects. *J. Agric. Food Chem.* **2000**, *48*, 2313-2319.
239. Yasukawa, K.; Akihisa, T.; Kimura, Y.; Tamura, T.; Takido, M. Inhibitory effect of cycloartenol ferulate, a component of rice bran, on tumor promotion in two-stage carcinogenesis in mouse skin. *Biol. Pharm. Bull.* **1998**, *21*, 1072-1076.
240. Baskar, A.A.; Al Numair, K.S.; Gabriel Paulraj, M.; Alsaif, M.A.; Muamar, M.A.; Ignacimuthu, S. β-sitosterol prevents lipid peroxidation and improves antioxidant status and histoarchitecture in rats with 1, 2-dimethylhydrazine-induced colon cancer. *J. Med. Food* **2012**, *15*, 335-343.
241. Kanehisa, M.; Goto, S. KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Res.* **2000**, *28*, 27-30.
242. Karlsson, P.C.; Huss, U.; Jenner, A.; Halliwell, B.; Bohlin, L.; Rafter, J.J. Human fecal water inhibits COX-2 in colonic HT-29 cells: role of phenolic compounds. *J. Nutr.* **2005**, *135*, 2343-2349.
243. Wang, T.; Hicks, K.B.; Moreau, R. Antioxidant activity of phytosterols, oryzanol, and other phytosterol conjugates. *J. Am. Oil Chem. Soc.* **2002**, *79*, 1201-1206.
244. Markiewicz, L.H.; Honke, J.; Haros, M.; Świątecka, D.; Wróblewska, B. Diet shapes the ability of human intestinal microbiota to degrade phytate–in vitro studies. *Journal of Applied Microbiology* **2013**, *115*, 247-259.

245. Lee, J.H.; Lee, J. Indole as an intercellular signal in microbial communities. *FEMS Microbiology Reviews* **2010**, *34*, 426-444.
246. Braune, A.; Bunzel, M.; Yonekura, R.; Blaut, M. Conversion of dehydrodiferulic acids by human intestinal microbiota. *J. Agric. Food Chem.* **2009**, *57*, 3356-3362.
247. Vetrani, C.; Rivellese, A.A.; Annuzzi, G.; Mattila, I.; Meudec, E.; Hyötyläinen, T.; Orešič, M.; Aura, A.-M. Phenolic metabolites as compliance biomarker for polyphenol intake in a randomized controlled human intervention. *Food Res. Int.* **2014**.
248. Gingras, D.; Béliveau, R. Colorectal cancer prevention through dietary and lifestyle modifications. *Cancer Microenvironment* **2011**, *4*, 133-139.
249. Bingham, S. Diet and colorectal cancer prevention. *Biochem. Soc. Trans.* **2000**, *28*, 12-16.
250. Marshall, J.R. Prevention of colorectal cancer: diet, chemoprevention, and lifestyle. *Gastroenterology clinics of North America* **2008**, *37*, 73-82.
251. Bobe, G.; Barrett, K.G.; Mentor-Marcel, R.A.; Saffiotti, U.; Young, M.R.; Colburn, N.H.; Albert, P.S.; Bennink, M.R.; Lanza, E. Dietary cooked navy beans and their fractions attenuate colon carcinogenesis in azoxymethane-induced ob/ob mice. *Nutr. Cancer* **2008**, *60*, 373-381.
252. Egeberg, R.; Olsen, A.; Loft, S.; Christensen, J.; Johnsen, N.; Overvad, K.; Tjønneland, A. Intake of wholegrain products and risk of colorectal cancers in the Diet, Cancer and Health cohort study. *Br. J. Cancer* **2010**, *103*, 730-734.
253. Fung, T.T.; Hu, F.B.; Wu, K.; Chiuve, S.E.; Fuchs, C.S.; Giovannucci, E. The Mediterranean and Dietary Approaches to Stop Hypertension (DASH) diets and colorectal cancer. *The American journal of clinical nutrition* **2010**, *92*, 1429-1435.
254. Lanza, E.; Hartman, T.J.; Albert, P.S.; Shields, R.; Slattery, M.; Caan, B.; Paskett, E.; Iber, F.; Kikendall, J.W.; Lance, P. High dry bean intake and reduced risk of advanced colorectal adenoma recurrence among participants in the polyp prevention trial. *J. Nutr.* **2006**, *136*, 1896-1903.
255. *Continuous Update Project colorectal cancer report 2010 summary. Food, nutrition, physical activity, and the prevention of colorectal cancer.* . World Cancer Research Fund / American Institute for Cancer Research: Washington DC: WCRF, 2011 May.
256. Huber, B.; Eberl, L.; Feucht, W.; Polster, J. Influence of polyphenols on bacterial biofilm formation and quorum-sensing. *Zeitschrift für Naturforschung C* **2003**, *58*, 879-884.
257. Lattanzio, V.; Lattanzio, V.M.; Cardinali, A. Role of phenolics in the resistance mechanisms of plants against fungal pathogens and insects. *Phytochemistry: Advances in research* **2006**, *661*, 23-67.
258. Tuohy, K.M.; Conterno, L.; Gasperotti, M.; Viola, R. Up-regulating the human intestinal microbiome using whole plant foods, polyphenols, and/or fiber. *J. Agric. Food Chem.* **2012**, *60*, 8776-8782.
259. Kelly, C.J.; Zheng, L.; Campbell, E.L.; Saeedi, B.; Scholz, C.C.; Bayless, A.J.; Wilson, K.E.; Glover, L.E.; Kominsky, D.J.; Magnuson, A. Crosstalk between microbiota-derived short-chain fatty acids and intestinal epithelial HIF augments tissue barrier function. *Cell host & microbe* **2015**, *17*, 662-671.
260. Sheflin, A.M.; Borresen, E.C.; Wdowik, M.J.; Rao, S.; Brown, R.J.; Heuberger, A.L.; Broeckling, C.D.; Weir, T.L.; Ryan, E.P. Pilot dietary intervention with heat-stabilized rice bran modulates stool microbiota and metabolites in healthy adults. *Nutrients* **2015**, *7*, 1282-1300, 10.3390/nu7021282.

261. Borresen, E.C.; Brown, D.G.; Harbison, G.; Taylor, L.; Fairbanks, A.; O'Malia, J.; Bazan, M.; Rao, S.; Bailey, S.M.; Wdowik, M.J.*, et al.* A randomized-controlled trial to increase navy bean or rice bran consumption in colorectal cancer survivors. *Nutr. Cancer* **2016**, (in press).
262. Langille, M.G.; Zaneveld, J.; Caporaso, J.G.; McDonald, D.; Knights, D.; Reyes, J.A.; Clemente, J.C.; Burkepile, D.E.; Thurber, R.L.V.; Knight, R. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat. Biotechnol.* **2013**, *31*, 814-821.
263. Lang, J.M.; Eisen, J.A.; Zivkovic, A.M. The microbes we eat: abundance and taxonomy of microbes consumed in a day's worth of meals for three diet types. *PeerJ* **2014**, *2*, e659.
264. Parks, D.H.; Tyson, G.W.; Hugenholtz, P.; Beiko, R.G. STAMP: statistical analysis of taxonomic and functional profiles. *Bioinformatics* **2014**, *30*, 3123-3124.
265. White, J.R.; Nagarajan, N.; Pop, M. Statistical methods for detecting differentially abundant features in clinical metagenomic samples. *PLoS Comput Biol* **2009**, *5*, e1000352.
266. Cardona, F.; Andrés-Lacueva, C.; Tulipani, S.; Tinahones, F.J.; Queipo-Ortuño, M.I. Benefits of polyphenols on gut microbiota and implications in human health. *J. Nutr. Biochem.* **2013**, *24*, 1415-1422.
267. Shade, A.; Peter, H.; Allison, S.D.; Baho, D.L.; Berga, M.; Bürgmann, H.; Huber, D.H.; Langenheder, S.; Lennon, J.T.; Martiny, J.B. Fundamentals of microbial community resistance and resilience. **2012**.
268. Sleeth, M.L.; Thompson, E.L.; Ford, H.E.; Zac-Varghese, S.E.; Frost, G. Free fatty acid receptor 2 and nutrient sensing: a proposed role for fibre, fermentable carbohydrates and short-chain fatty acids in appetite regulation. *Nutrition research reviews* **2010**, *23*, 135-145.
269. Wolever, T.; Spadafora, P.; Eshuis, H. Interaction between colonic acetate and propionate in humans. *The American journal of clinical nutrition* **1991**, *53*, 681-687.
270. Larsbrink, J.; Rogers, T.E.; Hemsworth, G.R.; McKee, L.S.; Tauzin, A.S.; Spadiut, O.; Klinter, S.; Pudlo, N.A.; Urs, K.; Koropatkin, N.M. A discrete genetic locus confers xyloglucan metabolism in select human gut Bacteroidetes. *Nature* **2014**, *506*, 498-502.
271. Shibuya, N.; Iwasaki, T. Structural features of rice bran hemicellulose. *Phytochemistry* **1985**, *24*, 285-289.
272. Louis, P.; Scott, K.; Duncan, S.; Flint, H. Understanding the effects of diet on bacterial metabolism in the large intestine. *Journal of applied microbiology* **2007**, *102*, 1197-1208.
273. Ley, R.E.; Bäckhed, F.; Turnbaugh, P.; Lozupone, C.A.; Knight, R.D.; Gordon, J.I. Obesity alters gut microbial ecology. *Proceedings of the National Academy of Sciences of the United States of America* **2005**, *102*, 11070-11075.
274. Sanapareddy, N.; Legge, R.M.; Jovov, B.; McCoy, A.; Burcal, L.; Araujo-Perez, F.; Randall, T.A.; Galanko, J.; Benson, A.; Sandler, R.S. Increased rectal microbial richness is associated with the presence of colorectal adenomas in humans. *ISME J.* **2012**, *6*, 1858-1868.
275. Neyrinck, A.M.; Possemiers, S.; Druart, C.; Van de Wiele, T.; De Backer, F.; Cani, P.D.; Larondelle, Y.; Delzenne, N.M. Prebiotic effects of wheat arabinoxylan related to the increase in bifidobacteria, Roseburia and Bacteroides/Prevotella in diet-induced obese mice. *PLoS One* **2011**, *6*, e20944.

276. Larsson, S.C.; Wolk, A. Obesity and colon and rectal cancer risk: a meta-analysis of prospective studies. *The American journal of clinical nutrition* **2007**, *86*, 556-565.
277. Shiga, T.M.; Lajolo, F.M. Cell wall polysaccharides of common beans (Phaseolus vulgaris L.)—Composition and structure. *Carbohydrate Polymers* **2006**, *63*, 1-12.
278. Cotta, M.; Forster, R. The family Lachnospiraceae, including the genera Butyrivibrio, Lachnospira and Roseburia. In *The Prokaryotes*, Springer: 2006; pp 1002-1021.
279. Vos, P.; Garrity, G.; Jones, D.; Krieg, N.R.; Ludwig, W.; Rainey, F.A.; Schleifer, K.-H.; Whitman, W. *Bergey's Manual of Systematic Bacteriology: Volume 3: The Firmicutes*. Springer Science & Business Media: 2011; Vol. 3.
280. Samuel, B.S.; Gordon, J.I. A humanized gnotobiotic mouse model of host–archaeal–bacterial mutualism. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 10011-10016.
281. Flint Hj Fau - Bayer, E.A.; Bayer, E.A. Plant cell wall breakdown by anaerobic microorganisms from the Mammalian digestive tract.
282. Ze, X.; Duncan, S.H.; Louis, P.; Flint, H.J. Ruminococcus bromii is a keystone species for the degradation of resistant starch in the human colon. *ISME J.* **2012**, *6*, 1535-1543.
283. Dwek, R.A. Glycobiology: toward understanding the function of sugars. *Chemical Reviews* **1996**, *96*, 683-720.
284. Srisuma, N.; Ruengsakulrach, S.; Uebersax, M.A.; Bennink, M.R.; Hammerschmidt, R. Cell wall polysaccharides of navy beans (Phaseolus vulgaris). *J. Agric. Food Chem.* **1991**, *39*, 855-858.
285. Martens, E.C.; Lowe, E.C.; Chiang, H.; Pudlo, N.A.; Wu, M.; McNulty, N.P.; Abbott, D.W.; Henrissat, B.; Gilbert, H.J.; Bolam, D.N. Recognition and degradation of plant cell wall polysaccharides by two human gut symbionts. *PLoS Biol.* **2011**, *9*, e1001221.
286. Koropatkin, N.M.; Cameron, E.A.; Martens, E.C. How glycan metabolism shapes the human gut microbiota. *Nature Reviews Microbiology* **2012**, *10*, 323-335.
287. Fuhrer, A.; Sprenger, N.; Kurakevich, E.; Borsig, L.; Chassard, C.; Hennet, T. Milk sialyllactose influences colitis in mice through selective intestinal bacterial colonization. *The Journal of experimental medicine* **2010**, *207*, 2843-2854.
288. Chaturvedi, P.; Warren, C.D.; Buescher, C.R.; Pickering, L.K.; Newburg, D.S. Survival of human milk oligosaccharides in the intestine of infants. In *Bioactive components of human milk*, Springer: 2001; pp 315-323.
289. German, J.; Freeman, S.; Lebrilla, C.; Mills, D. Human milk oligosaccharides: evolution, structures and bioselectivity as substrates for intestinal bacteria. **2008**.
290. Gnoth, M.J.; Kunz, C.; Kinne-Saffran, E.; Rudloff, S. Human milk oligosaccharides are minimally digested in vitro. *J. Nutr.* **2000**, *130*, 3014-3020.
291. Bäckhed, F.; Ley, R.E.; Sonnenburg, J.L.; Peterson, D.A.; Gordon, J.I. Host-bacterial mutualism in the human intestine. *Science* **2005**, *307*, 1915-1920.
292. Marcobal, A.; Southwick, A.M.; Earle, K.A.; Sonnenburg, J.L. A refined palate: bacterial consumption of host glycans in the gut. *Glycobiology* **2013**, *23*, 1038-1046.
293. Mahowald, M.A.; Rey, F.E.; Seedorf, H.; Turnbaugh, P.J.; Fulton, R.S.; Wollam, A.; Shah, N.; Wang, C.; Magrini, V.; Wilson, R.K. Characterizing a model human gut microbiota composed of members of its two dominant bacterial phyla. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 5859-5864.
294. Tosh, S.M.; Yada, S. Dietary fibres in pulse seeds and fractions: Characterization, functional attributes, and applications. *Food Res. Int.* **2010**, *43*, 450-460.

295. Salyers, A.; Vercellotti, J.; West, S.; Wilkins, T. Fermentation of mucin and plant polysaccharides by strains of Bacteroides from the human colon. *Appl. Environ. Microbiol.* **1977**, *33*, 319-322.
296. Ley, R.E. Obesity and the human microbiome. *Current opinion in gastroenterology* **2010**, *26*, 5-11.
297. Cummings, J.H.; Macfarlane, G.T. The control and consequences of bacterial fermentation in the human colon. *Journal of Applied Microbiology* **1991**, *70*, 443-459, 10.1111/j.1365-2672.1991.tb02739.x.
298. Weaver, G.; Krause, J.; Miller, T.; Wolin, M. Short chain fatty acid distributions of enema samples from a sigmoidoscopy population: an association of high acetate and low butyrate ratios with adenomatous polyps and colon cancer. *Gut* **1988**, *29*, 1539-1543.
299. Ono, S.; Karaki, S.-i.; Kuwahara, A. Short-chain fatty acids decrease the frequency of spontaneous contractions of longitudinal muscle via enteric nerves in rat distal colon. *The Japanese journal of physiology* **2004**, *54*, 483-493.
300. Tedelind, S.; Westberg, F.; Kjerrulf, M.; Vidal, A. Anti-inflammatory properties of the short-chain fatty acids acetate and propionate: a study with relevance to inflammatory bowel disease. *World journal of gastroenterology: WJG* **2007**, *13*, 2826-2832.
301. Hinnebusch, B.F.; Meng, S.; Wu, J.T.; Archer, S.Y.; Hodin, R.A. The effects of short-chain fatty acids on human colon cancer cell phenotype are associated with histone hyperacetylation. *J. Nutr.* **2002**, *132*, 1012-1017.
302. Zapolska-Downar, D.; Naruszewicz, M. Propionate reduces the cytokine-induced VCAM-1 and ICAM-1 expression by inhibiting nuclear factor-kappa B (NF-kappaB) activation. *J Physiol Pharmacol* **2009**, *60*, 123-131.
303. Jan, G.; Belzacq, A.; Haouzi, D.; Rouault, A.; Metivier, D.; Kroemer, G.; Brenner, C. Propionibacteria induce apoptosis of colorectal carcinoma cells via short-chain fatty acids acting on mitochondria. *Cell Death Differ.* **2002**, *9*, 179-188.
304. Fukuda, S.; Toh, H.; Hase, K.; Oshima, K.; Nakanishi, Y.; Yoshimura, K.; Tobe, T.; Clarke, J.M.; Topping, D.L.; Suzuki, T. Bifidobacteria can protect from enteropathogenic infection through production of acetate. *Nature* **2011**, *469*, 543-547.
305. Rombeau, J.L.; Kripke, S.A. Metabolic and intestinal effects of short-chain fatty acids. *JPEN. Journal of parenteral and enteral nutrition* **1989**, *14*, 181S-185S.
306. Tolhurst, G.; Heffron, H.; Lam, Y.S.; Parker, H.E.; Habib, A.M.; Diakogiannaki, E.; Cameron, J.; Grosse, J.; Reimann, F.; Gribble, F.M. Short-chain fatty acids stimulate glucagon-like peptide-1 secretion via the G-protein–coupled receptor FFAR2. *Diabetes* **2012**, *61*, 364-371.
307. Freeland, K.R.; Wilson, C.; Wolever, T.M. Adaptation of colonic fermentation and glucagon-like peptide-1 secretion with increased wheat fibre intake for 1 year in hyperinsulinaemic human subjects. *Br. J. Nutr.* **2010**, *103*, 82-90.
308. Tarini, J.; Wolever, T.M. The fermentable fibre inulin increases postprandial serum short-chain fatty acids and reduces free-fatty acids and ghrelin in healthy subjects. *Appl. Physiol. Nutr. Metab.* **2010**, *35*, 9-16.
309. Gribble, F. RD Lawrence Lecture 2008 Targeting GLP−1 release as a potential strategy for the therapy of Type 2 diabetes. *Diabetic Medicine* **2008**, *25*, 889-894.
310. Windey, K.; De Preter, V.; Verbeke, K. Relevance of protein fermentation to gut health. *Mol. Nutr. Food Res.* **2012**, *56*, 184-196.

311. Halton, T.L.; Hu, F.B. The effects of high protein diets on thermogenesis, satiety and weight loss: a critical review. *J. Am. Coll. Nutr.* **2004**, *23*, 373-385.
312. O'Neil, C.E.; Keast, D.R.; Fulgoni, V.L.; Nicklas, T.A. Food sources of energy and nutrients among adults in the US: NHANES 2003–2006. *Nutrients* **2012**, *4*, 2097-2120.
313. Clemens, R.; Kranz, S.; Mobley, A.R.; Nicklas, T.A.; Raimondi, M.P.; Rodriguez, J.C.; Slavin, J.L.; Warshaw, H. Filling America's fiber intake gap: summary of a roundtable to probe realistic solutions with a focus on grain-based foods. *J. Nutr.* **2012**, *142*, 1390S-1401S.
314. Ridlon, J.M.; Kang, D.-J.; Hylemon, P.B. Bile salt biotransformations by human intestinal bacteria. *Journal of lipid research* **2006**, *47*, 241-259.
315. Kahlon, T.; Woodruff, C. In vitro binding of bile acids by rice bran, oat bran, barley and β-glucan enriched barley. *Cereal Chemistry* **2003**, *80*, 260-263.