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Fighting Cancer with Functional Foods: New Approaches to Investigate the Interactions of Dietary Bioactive Chemicals and the Gut Microbiome

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Cancer is a leading cause of death worldwide. The Western dietary pattern is an established risk factor for many cancers, particularly for colorectal cancer (CRC). The Western diet is typified by the high consumption of red and processed meats, high fat foods, sugary foods and refined grains, whereas a more prudent diet replaces these foods with whole grains, fruits and vegetables, many of which are rich in dietary bioactives known to reduce cancer risk. Agricultural production of many of the foods common to the Western diet is also estimated to have a high environmental impact. Thus, diet modification to reduce cancer risk by consumption of more fruits and vegetables would also be considered a more environmentally sustainable diet.

This review summarizes the impact of dietary bioactives on gastrointestinal health, with a focus on the role of the gut microbiome and intestinal inflammation in colorectal carcinogenesis. Four dietary bioactives with purported anti-cancer activities are discussed, including catechins (green tea), anthocyanins (red/blue berries), proanthocyanidins (cocoa) and isoflavones (soy), with special consideration given to evidence for their interaction with the gut microbiome. The review concludes with a proposed model for investigating the impact of dietary bioactives for prevention of colon cancer that incorporates the Western nutritional pattern and considers the role of human gut microbiota in pre-clinical studies.

Key words: Colon cancer, dietary bioactives, flavonoids, gut microbiome, western diet

1. Introduction

In recent years, scientists and policy makers have become increasingly concerned with the problem of sustainable production of high quality, nutritious food (Burchi *et al*., 2011; O' Kane, 2012; Institute of Medicine, 2014). The consensus of these reports is that, in the 21st century, it is not enough to produce food in sufficient quantity to meet caloric needs of the world's population. Food must also meet nutritional needs, especially with respect to its micronutrient and bioactive chemical content (i.e., minerals, vitamins and other food-derived chemicals that affect health). Recently, the Barilla Center for Food and Nutrition (BCFN, 2014) finalized its "Double Pyramid" model, described as a "unique food model created to protect the wellbeing of people and the environment" (Fig. 1). The food pyramid depicts recommended dietary intakes of foods based on a prudent dietary pattern (modeled after the Mediterranean diet), which is known to promote health and reduce risk of various chronic diseases. Alternatively, the environmental

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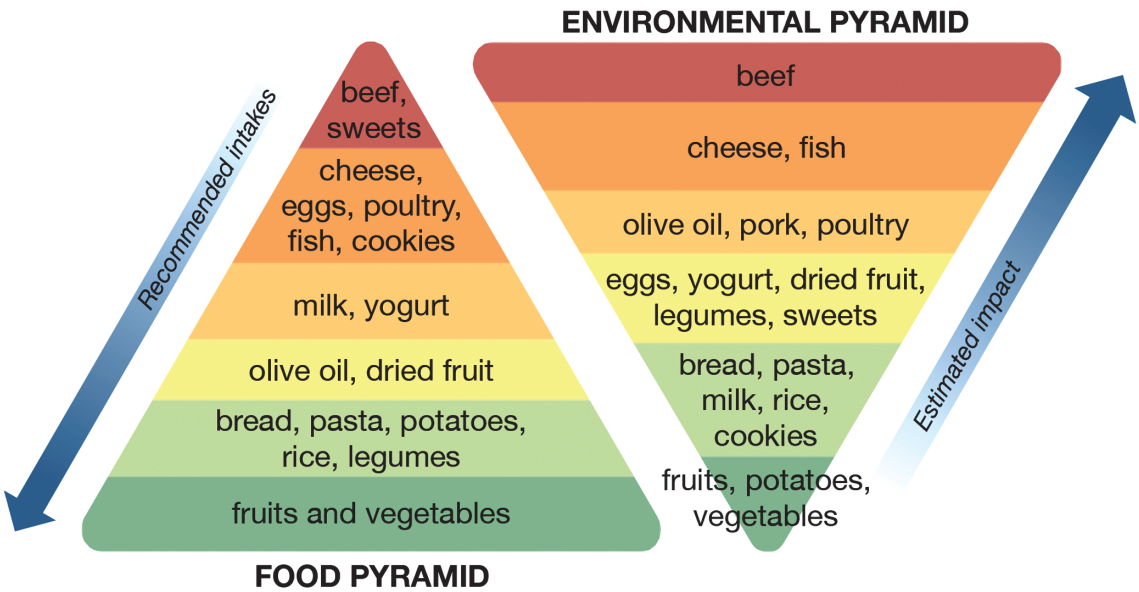


Fig. 1. This diagram represents a simplified version of the "Double Pyramid" (Barilla Center for Food and Nutrition, 2014), which compares foods ranked in order of recommended intakes for optimal health to foods ranked in order of estimated environmental impact associated with their production.

pyramid represents the estimated environmental impact associated with production of these foods, ranked from lowest to highest impact. Of critical importance is the observation that the food items that are recommended at the highest intakes for optimal health, including fruits and vegetables, have the lowest estimated environmental impact.

Many developed nations, including the United States, are typified by a pattern of food consumption that conflicts with this double pyramid model. Americans tend to consume high amounts of red meats, processed meats, sweets, high fat foods, refined grains, high sugar drinks and high fat dairy products – items that have substantial environmental impact and relatively moderate to low nutritional value. Moreover, the Western dietary pattern is associated with increased risk of many diseases, including diabetes, obesity, hypertension, cancer, autoimmune disease, cardiovascular disease, and fatty liver disease. Logic suggests that diet modification represents a safe and effective strategy to reduce risk of these "Western" diseases. This strategy has the added societal benefit in that many of the foods that are believed to reduce disease risk, particularly fruits and vegetables, are also those that have low estimated environmental impact.

This review focuses on the impact of dietary bioactives on gastrointestinal health, a priority research topic for the Agricultural Food and Research Initiative with the U.S. Department of Agriculture and the principal research area of the Applied Nutrition Research team at Utah State University. This report reviews critical statistics on colon cancer risk worldwide, the impact of diet on cancer, and the role of the gut microbiome and inflammation in development of colorectal cancer. We also discuss dietary bioactives for cancer prevention, with a focus on selected bioactives that have been shown to reduce risk of colon cancer and impact the gut microbiome. Finally, we highlight two methodological advances that have allowed us to overcome key challenges facing researchers engaged in pre-clinical research to address the impact of diet on gut health: 1) a new defined diet that better emulates typical Western nutrition for rodent animal models and 2) a new strategy for humanizing the gut microbiome of rodents. The review will conclude with a proposed model for investigating the impact of dietary bioactives for prevention of colon cancer that incorporates the Western nutritional pattern and considers the role of human gut microbiota in pre-clinical studies.

2. Colorectal cancer

Cancer is a leading cause of death worldwide, with approximately 8.2 million deaths reported for 2012

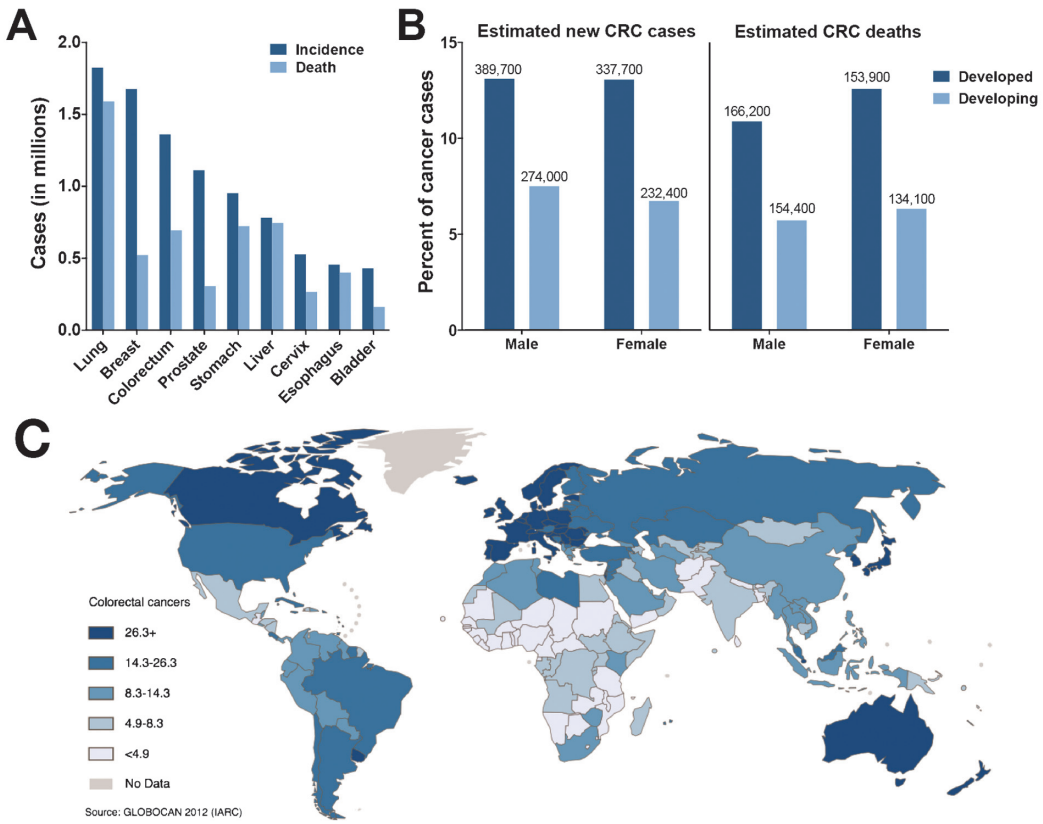


Fig. 2. Estimated number of cases for the most common cancers worldwide in 2012 (most recent data available). B) Bars represent the estimated number of colorectal cancer (CRC) cases and the estimated number of CRC deaths as a percentage of all cancers (excluding non-melanoma skin cancers) for male or females in developed (dark blue) or developing (light blue) nations. Numbers above the bar represent the number of cases or deaths. C) World map depicting incidence of colorectal cancers by nation for both males and females (values shown are the age-standardized rate per 100,000 people). Source data for panels A and B were obtained from the International Agency for Research on Cancer (2014). Source data for panel C were obtained from the GLOBOCAN 2012 database (Ferlay *et al*., 2013).

(International Agency for Research on Cancer, 2014). Leading causes of cancer deaths worldwide include lung, breast, colorectal, prostate, stomach, and liver cancers (Fig. 2A). Approximately 66% of new cancer diagnoses are for patients that reside in countries that are economically developed, whereas 53% of cancer-related deaths occur in countries that economically underdeveloped or developing. Moreover, the pattern of dominant cancers differs according to economic development status, with breast, prostate, lung, colorectal and stomach cancers more prevalent in highly economically developed nations and breast, cervix, prostate, liver and esophageal cancers more common in countries with low economic development. Scientists predict that improvement in economic status may cause a shift in this disease profile as countries become more "Westernized," thus resulting in fewer cancers caused by chronic infections and leading to a higher burden of reproductive cancers and diseases associated with diet and hormonal risk factors.

Colorectal cancer (CRC) is the third most commonly diagnosed cancer world-wide, with an estimated 1.2 million new cases diagnosed in 2012. A clear disparity in rates of CRC is evident when comparing developed and developing countries (Fig. 2B, C), as CRC cases account for about 13% of new diagnoses (excluding skin cancers) in developed countries (e.g., North America) compared to only 7% of new cases diagnosed in developing countries (e.g., Sub-Saharan Africa) (American Cancer Society, 2011). In the U.S., CRC affects primarily those over the age of 50 and has the highest incidence in whites and African

Americans. The disease is classified as either hereditary or sporadic, according to etiology. Hereditary factors account for about 20% of all cases (Rustgi, 2007), including patients diagnosed with familial adenomatous polyposis who harbor a mutation in the adenomatous polyposis coli (APC) gene. Alternatively, sporadic CRC is attributed to environmental and lifestyle factors, such as diet, physical activity, obesity, smoking and excess alcohol intake.

A major risk factor for development of CRC is the presence of chronic inflammation in the colon, which occurs in patients with inflammatory bowel disease (IBD). An estimated 1.4 million people suffer from IBD in the U. S. (Loftus, 2004), including patients diagnosed with ulcerative colitis (UC) and Crohn's disease. Genetic, environmental, lifestyle and immunological factors are believed to contribute to the development and progression of IBD. The prognosis for sporadic and IBD-associated CRC is similar, with survival at five years estimated to be about 50% (Rhodes and Campbell, 2002). Importantly, researchers have identified clear links between colon inflammation and increased risk of neoplasia in the colon mucosa (Dyson and Rutter, 2012; Grivennikov, 2013 and references therein). IBD patients with prolonged colitis, pan-colitis (involving the whole large bowel), and severe inflammation are at greatest risk of developing CRC. Treatment with anti-inflammatory drugs reduces the risk of developing IBD-associated CRC, an observation that is consistent with the involvement of inflammation in colon carcinogenesis (Ullman and Itzkowitz, 2011 and references therein). Rutter, *et al*. (2006) made the critical observation that recovery from colitis in IBD patients restored their cancer risk level to that of the general population. Thus, intervention strategies to enhance recovery from colonic inflammation could markedly reduce risk of progression to CRC.

Evidence from animal studies has shown that prolonged chronic inflammation, caused by chemical injury or by infections that induce colitis, can trigger DNA damage and colon tumorigenesis (Meira *et al*., 2008; Boulard *et al*., 2012; Mangerich *et al*., 2012). Under conditions of inflammation, reactive oxygen and nitrogen species generated by cells of the innate immune system also play an important role in triggering genetic and epigenetic changes to colon epithelial cells, leading to initiation and/or promotion of tumorigenesis (Hussain *et al*., 2003). Collectively, this evidence suggests that inflammation can functionally bypass the initial mutation step, typically to the *APC* gene, to initiate colorectal carcinogenesis under conditions of colitis. Moreover, this evidence infers that cancer progression could be arrested and tissue repair achieved if the inflammatory conditions responsible for the aberrant signaling driving inappropriate growth and proliferation of intestinal epithelial cells are resolved.

3. The Western diet, dietary bioactives and mechanisms of cancer prevention

Approximately one quarter of all deaths in countries with a Westernized lifestyle are attributed to cancer (Boyle and Langman, 2000). The Western dietary pattern is characterized by high intakes of red and processed meats, sweets, fried foods and refined grains, whereas a more balanced diet replaces these foods with fruits and vegetables, legumes, fish, poultry and whole grains. In case-controlled and cohort studies, the typical Western diet is associated with significantly higher rates of colorectal cancer (CRC) compared to a balanced diet (Meyerhardt *et al*., 2007); environmental factors may contribute approximately 70% of this risk (Doll and Peto, 1981; Wiseman, 2008; Jemal *et al*., 2009). The World Health Organization states that "prevention offers the most cost-effective long-term strategy for the control of cancer" (World Health Organization). Regular physical activity, maintenance of a healthy body weight and consumption of a balanced diet may considerably reduce cancer risk.

Diet modification represents a safe and cost-effective strategy to decrease the incidence of cancer and delay the onset of the disease. A number of foods have been identified that may reduce cancer risk with regular consumption, including certain fruits, vegetables and whole grains. These plant-derived foods contain a variety of components, including vitamins and minerals, polyunsaturated fatty acids, and various phytochemicals, that can influence the molecular, cellular or systemic physiology of the consumer. Collectively, these essential and non-essential compounds are referred to as dietary bioactives. However, many individuals consume a diet that is deficient in these food items and the beneficial micronutrients and bioactive compounds they provide; the typical Western diet is emblematic of this problem. For example, consumption of vegetables and micronutrients such as vitamins B_6, B_{12}, D, C and E as well as folate, omega-

FLAVONOIDS

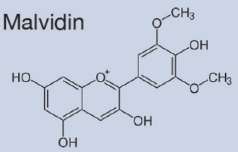
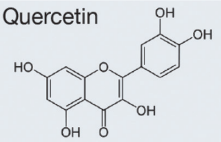
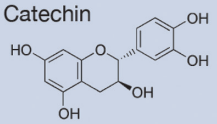
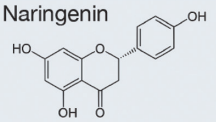
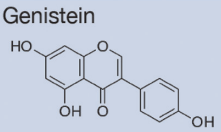
SUBCLASS	EXAMPLES	FOOD SOURCES	STRUCTURE
Anthocyanidins	Cyanidin, delphinidin, malvidin, peragonidin, peonidin, petunidin	Red, blue and purple berries; red and purple grapes, red wine	Malvidin 
Flavonols	Quercetin, kaempferol, myricetin	Yellow onions, scallions, kale, broccoli, apples, berries, teas	Quercetin 
Flavanols	catechins, epicatechin, epigallocatechin, epigallocatechin gallate, theaflavins, therubigins, proanthocyanidins	Teas, chocolate, grapes, berries, apples, red wine	Catechin 
Flavanones	Hesperetin, naringenin, eriodictyol	Citrus fruits and juices	Naringenin 
Isoflavones	Daidzein, genistein, glycitein	Soybeans, soy foods, legumes	Genistein 

Fig. 3. Subclasses of dietary flavonoids, including anthocyanidins, flavonols, flavanols, flavanones and isoflavones, with example bioactives, some common food sources and a representative chemical structure for each subclass.

3 polyunsaturated fatty acids, calcium and selenium have been linked to decreased risk of colon cancer in humans (Roynette *et al*., 2004; Kune and Watson, 2006; Kim and Milner, 2007; Forte *et al*., 2008; Pufulete, 2008; Larsson *et al*., 2010). To date, hundreds of dietary bioactives have been identified with proven or suggested beneficial health effects, including cancer prevention. Many of these compounds are plant-derived chemicals (often referred to as "botanicals") in the polyphenol chemical class with a flavonoid-based structure (Fig. 3). Example source foods for flavonoids include green tea, various berries (strawberries, black berries, blueberries, raspberries), pigmented grains (purple corn), beans (black and kidney beans), nuts (walnuts, almonds), apples, artichokes, broccoli, kale, soybean, grapes and grape juices.

Carcinogenesis is generally considered a multi-step process, wherein multiple changes to the genetic code and/or function of cancer critical genes are required to induce abnormal growth and proliferation of cells, including processes associated with carcinogen metabolism, DNA repair, epigenome modification, cell cycle regulation, apoptosis, and inflammation (see reviews by Davis, 2007; Sarkar and Li, 2007). Thus, consumption of dietary bioactives, such as many of those in the flavonoid group, that function to restore appropriate cellular signaling by correcting epigenetic errors, improving DNA repair, inducing cell cycle arrest or triggering apoptosis in defective cells, may decrease cancer risk (summarized in Fig. 4). Of particular interest in the case of colorectal cancer is inflammation, which occurs as a normal physiological response to pathogens, irritation or tissue injury.

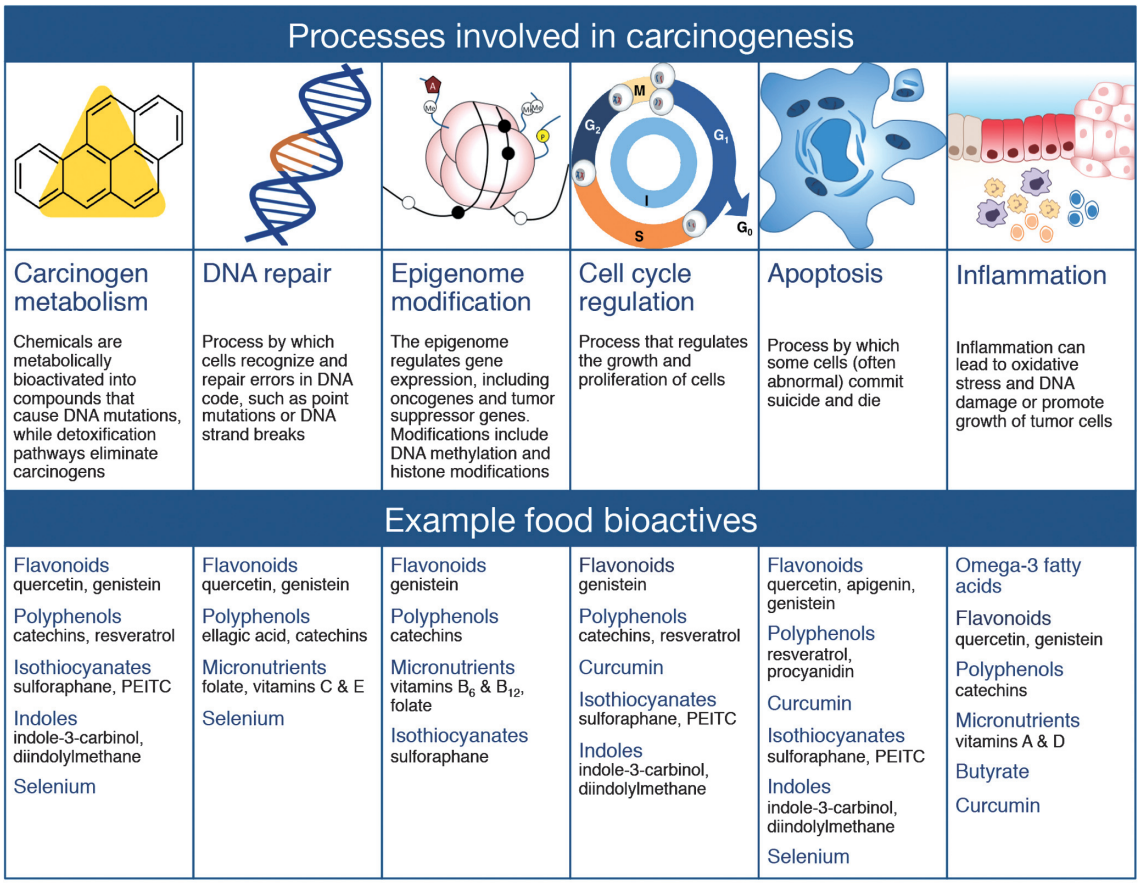


Fig. 4. Bioactive food compounds can target a variety of cellular processes that are involved in carcinogenesis, including carcinogen metabolism, DNA repair, epigenome modification, cell cycle regulation, apoptosis and inflammation. Also shown are selected bioactive compounds that have been shown to modulate these cellular processes to prevent or suppress cancer development. Selected compounds are indicated, although this list is not exhaustive.

While acute inflammation can be beneficial to the organism by aiding healing, chronic inflammation is often detrimental. Chronic inflammation of colon tissues leads to increased DNA damage, disruption of DNA repair, aberrant cell proliferation, reduced apoptosis, angiogenesis and invasion of malignant cells to other tissues. Dietary bioactives that suppress inflammation in the colon and/or the secondary effects of chronic inflammation on colonocytes may be effective in suppressing colon carcinogenesis.

4. The gut microbiome and its role in health and disease

Through the concerted action of the National Institute of Health's Human Microbiome Project, the European Commission's Metagenomics of the Human Intestinal Tract project and similar consortia across the world, a wealth of new knowledge has been gained on the impact of the gut microbiome on health and disease. Indeed, the number of diseases and conditions that may be influenced by the composition and metabolic activities of the gut microbiome is expansive, with new microbiome-disease connections reported frequently. Efforts directed towards identifying specific gut microbiome patterns that are associated with disease risk and/or pathology severity are ongoing and are providing new targets for risk reduction or therapy.

The human gut is host to an ecosystem of more than 100 trillion bacteria, which represent more than 1000 species-level phenotypes across the human population. Of these, about 160 species are prevalent in any one individual, and most are classified within two phyla, *Firmicutes* and *Bacteriodetes*. The gut metagenome

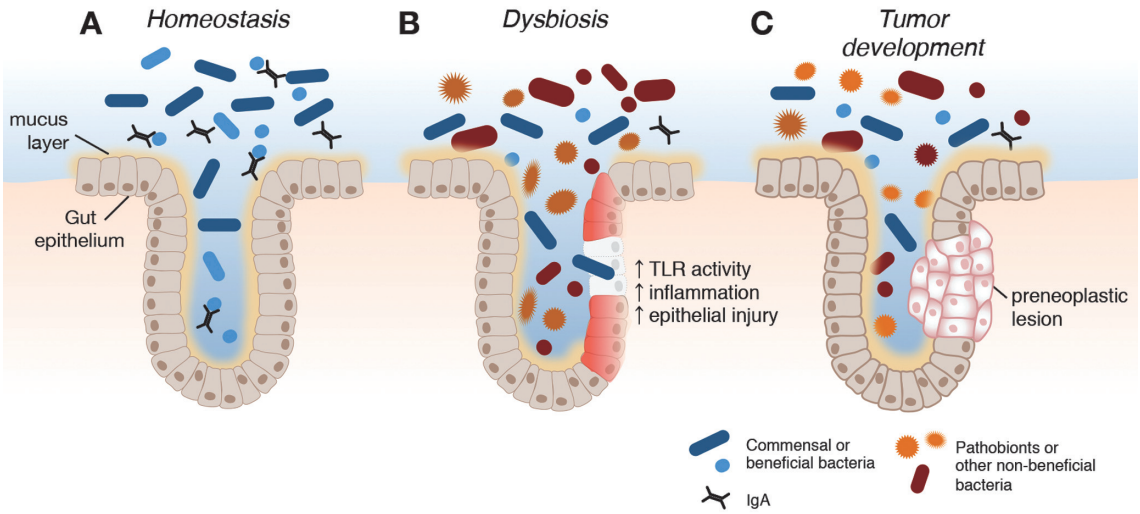


Fig. 5. A) Homeostasis between the gut microbiome and the intestinal epithelium exists when a beneficial bacteria population supports epithelial barrier function and a tolerant immune response. B) Triggered by genetic or environmental factors, such as diet or stress, dysbiosis can lead to loss of barrier function, translocation of commensal or pathogenic bacterial, dysregulation of immune response and inflammation of the intestinal epithelium. C) Excessive, chronic inflammation can promote hyperplasia and/or dysplasia of the intestinal epithelium and development of preneoplastic lesions, which may ultimately progress to form colon tumors.

consists of more than 3 million microbial genes, 150-fold more than that of the human genome (reviewed in Arthur and Jobin, 2011). The gut microbiome confers significant benefit to its host, including metabolism of indigestible compounds, energy production, defense against colonization by opportunistic pathogens and proper development and function of the gut immune system (reviewed in Round and Mazmanian, 2009). Changes to an individual's internal or external environment, including personal interactions, lifestyle, age and pathophysiology, can lead to changes in the gut microbiome composition and function. Gut microbiota modulate various physiological functions related to cancer development, including inflammation, cell proliferation, apoptosis and angiogenesis. Thus, it is likely that the gut microbiome directly affects colon tumorigenesis. Indeed, a recent report by Zackular, *et al*. (2013) showed that conventionalization of germ-free mice with gut microbiota from animals bearing colon tumors (generated using a model of inflammation-associated colorectal carcinogenesis) significantly increased colon tumorigenesis compared to mice conventionalized with bacteria from healthy animals. Importantly, antibiotic treatment caused a marked decrease in tumor number and size. The authors concluded that changes in the gut microbiome associated with inflammation and tumorigenesis directly contribute to colon tumorigenesis (Zackular *et al*., 2013). Recent studies have investigated the hypothesis that distinct microbiota populations are associated with CRC (Shen *et al*., 2010; Sobhani *et al*., 2011; Chen *et al*., 2012; Kostic *et al*., 2012; Wang *et al*., 2012; Ahn *et al*., 2013; Chen *et al*., 2013; Geng *et al*., 2013). Sobhani *et al*., (2011) found that microbiota from CRC patients clustered distinctly from matched, cancer free controls. Shen *et al*., (2010) also reported that adherent bacteria populations from CRC patients were significantly different from controls. Abundance of *Dorea* and *Faecalibacterium* species in CRC patients was higher compared to matched controls, whereas abundance of *Bacteroides* and *Coprococcus spp.* were lower. Additionally, individual bacterial species such as *Bacteroides fragilis* (Toprak *et al*., 2006; Wu *et al*., 2009), *Enterococcus faecalis* (Wang *et al*., 2008) and *Fusobacterium spp.* (McCoy *et al*., 2013) have all been implicated with increased CRC risk. From these reports, it is evident that CRC patients harbor a different gut microbiome compared to healthy individuals. However, results from these various studies do not agree with respect to

the composition and structure of the microbial community associated with CRC – a consensus cancer-related microbiome has not (yet) been identified.

Maladaptation to a changing environment can lead to dysbosis, or an imbalance in the structure and/or function of the gut microbiome. Under homeostatic conditions, symbiotic or commensal bacteria predominate, appropriately regulate the immune system and inhibit growth of pathobionts (Fig. 5). In patients with chronic inflammation, a shift in the microbiota population, triggered by a combination of genetic and environmental factors, can lead to dysregulation of the immune system, disruption of the epithelial barrier, increased production of pro-inflammatory and pro-tumorigenic cytokines, metabolic activation of various mutagens, loss of protective bacteria species and accumulation of opportunistic pathobionts (Grivennikov, 2013; Kamada *et al*., 2013). Translocation of bacteria to the submucosa leads to activation of pattern recognition receptors, such as toll-like receptors (TLRs), which in turn activate pro-inflammatory signaling cascades (e. g., NFκB pathway) leading to increased expression of pro-inflammatory cytokines (e.g., IL-1, IL-6, TNF) (Saleh and Trinchieri, 2011).

The gut microbiome can promote carcinogenesis through multiple mechanisms, such as the promotion of epithelial inflammation as described above (reviewed by Schwabe and Jobin, 2013). The gut microbiomes of patients with inflammation are distinct from healthy controls, with consistent observations of reduced gut microbial biomass, decreased diversity and richness of the microbial community and altered relative abundance of members of the dominant phyla, *Firmicutes* and *Bacteroidetes* (Ott *et al*., 2004; Frank *et al*., 2007; Ott *et al*., 2008). Inflammation of the intestine in colitis-associated CRC further alters the microbiome, selecting for overrepresentation of particular species. Furthermore, colon tumors may provide a specialized microenvironment that is suitable for colonization by certain species, such as *Fusobacterium spp.* (McCoy *et al*., 2013), which may function to further promote tumor development. Bacterial genotoxins can induce DNA damage in tissues of the gastrointestinal tract, leading to initiation of carcinogenesis. Reactive oxygen and nitrogen species released from inflammatory cells, such as macrophages, may also be genotoxic. The gut microbiome plays an important metabolic role in carcinogenesis, as well. Carcinogens consumed from the diet or dietary bioactives may undergo metabolic activation by the gut microbiome. Importantly, many of the metabolic products of the gut microbiome can exert both local and systemic effects.

5. Dietary bioactives and the gut microbiome.

Knowledge is accumulating regarding the impact of diet on the gut microbiome and is revealing dietary approaches to favorably affect the gut microbiome. To date, a substantial amount of effort has been directed towards the study of probiotics (live beneficial bacteria) and prebiotics (fermentable substrates) on the gut microbiome and the health issue of interest. Additional attention has been given to the role of macronutrients in defining both the gut microbiome and associated diet-derived metabolites (Wu *et al*., 2011; Ou *et al*., 2013; Daniel *et al*., 2014; David *et al*., 2014). In contrast to work done with prebiotics, probiotics and macronutrients, substantially less attention has been given to the potential for non-nutritive plant bioactive compound to alter both the gut microbiome and associated metabolic capabilities. The potential for dietary flavonoids to favorably alter the gut microbiome to promote health has been recognized and recently reviewed (Macdonald and Wagner, 2012; Tuohy *et al*., 2012; Etxeberria *et al*., 2013; Kemperman *et al*., 2013). Many flavonoids occur in plants as a defense mechanism against bacterial pathogens and thus have antibacterial properties (Cushnie and Lamb, 2005). Not surprisingly, studies conducted in animal models, humans and *in vitro* intestinal models demonstrate that the gut microbiota composition is altered by flavonoid-rich foods and extracts such as black tea, green tea, coffee, cocoa flavanols, cruciferous vegetables, blueberries, red wine polyphenols, or purified catechin and epicatechin (Mai *et al*., 2004; Dolara *et al*., 2005; Tzounis *et al*., 2008; Jaquet *et al*., 2009; Li *et al*., 2009; Tzounis *et al*., 2011; Axling *et al*., 2012; Hidalgo *et al*., 2012; Jin *et al*., 2012; Massot-Cladera *et al*., 2012; Queipo-Ortuno *et al*., 2012; Sanchez-Patan *et al*., 2012; Kemperman *et al*., 2013; Lacombe *et al*., 2013). Fig. 6 highlights selected food sources of dietary polyphenols and their effects on gut microbiota populations in humans and rodent models.

Addition of dietary bioactives, especially plant-derived polyphenols, to the American diet represents a safe and cost-effective strategy to reduce gut in-

Model	Bioactive source	o_Actinomycetales	g_Corynebacterium	f_Micrococcaceae	f_Nocardioidaceae	f_Bifidobacteriaceae	g_Bifidobacterium	f_Coriobacteriaceae	g_Eggerthella	g_Slackia	g_Bacteroides	g_Alistipes	g_Staphylococcus	g_Enterococcus	g_Lactobacillus	Clostridium histolyticum group	Clostridium/Eubacterium group	g_Blautia	f_Peptococcaceae	g_Subdoligranulum	p_Fusobacteria	g_Vicitvallis	g_Klebsiella	g_Cloacibacillus	g_Akkermansia	Footnote to reference in legend
Human	Red wine polyphenol						+		+					+				+			+					1
Human	Red wine polyphenol						-				-	+						-		-		+	+	+	+	2
Rat	Red wine polyphenol						+				+				+	-										3
Rat	Blueberries	+	+	+	+	+		+		+				-	-				+							4
Human	Anthocyanins						+								+											5
Human	Green tea extract						+																			6
Human	Cocoa catechins						+									-	+									7
Human	Cocoa						+							+	+	-										8
Rat	Cocoa										-		-			-										9

+ Increase in abundance - Decrease in abundance

Fig. 6. Evidence that select dietary bioactives modulate gut microbiota in humans and in pre-clinical animal models. [1](Queipo-Ortuno *et al*., 2012), [2](Kemperman *et al*., 2013), [3](Dolara *et al*., 2005), [4](Lacombe *et al*., 2013), [5](Hidalgo *et al*., 2012), [6](Jin *et al*., 2012), [7](Tzounis *et al*., 2008), [8](Tzounis *et al*., 2011) and [9](Massot-Cladera *et al*., 2012).

flammation, promote recovery from injury to the colon epithelium and decrease the risk of disease progression. Dietary polyphenols are extensively metabolized by intestinal microbiota. Only 5 to 10% of ingested polyphenols are absorbed in the small intestine (Clifford, 2004). Thus, the remaining 90 to 95% are metabolized in the colon by gut microbiota into numerous different chemical species (Gonthier *et al*., 2003; Rechner *et al*., 2004; Keppler and Humpf, 2005; Del Rio *et al*., 2010; Del Rio *et al*., 2010; Schantz *et al*., 2010; Van't Slot *et al*., 2010; Andres *et al*., 2011; van Duynhoven *et al*., 2011; Hidalgo *et al*., 2012; Moco *et al*., 2012; Bolca *et al*., 2013). As opposed to inactivation through microbial metabolism, pre-clinical data demonstrate that many of the known polyphenol metabolites retain anti-inflammation and anti-cancer bioactivities (Gao *et al*., 2006; Veeriah *et al*., 2007; Larrosa *et al*., 2009; Forester and Waterhouse, 2010; Miene *et al*., 2011; Russell and Duthie, 2011; Brown *et al*., 2012; Forester *et al*., 2012). Therefore, it is likely that the relationship between polyphenol intake and colon cancer risk reduction is more related to end products of microbial metabolism than the parent polyphenols consumed. Thus, protection against colon cancer by polyphenols may be dictated in part by the gut microbiota population and their metabolic capabilities. Below, we highlight several classes of dietary bioactives and present a summary of evidence for involvement of gut microbiota in their actions, with a focus on plant-derived polyphenols that have been shown to prevent or suppress colon carcinogenesis.

5.1 Green tea catechins

Green tea (*Camellia sinensis*) is the second most widely consumed beverage in the world and is one of the richest sources of dietary catechins ((-) -epicatechin; (-) -epicatechin 3-gallate; (-) -epigallocatechin; (-) -epigallocatechin 3-gallate; (+) -catechin; (+) -gallocatechin) (Singh *et al*., 2011). While other foods such as blueberries and cocoa approach green tea in terms of their content of total catechins, green tea is unique in its abundance of (-)-epigallocatechin 3-gallate (EGCG). Routine consumption of green tea has been linked to health benefits for multiple conditions, including cancer, obesity, stroke, diabetes, neurodegeneration and stress (reviewed in Singh *et al*., 2011). In addition to the availability of green tea for direct consumption as a beverage, numerous green tea extracts, purportedly high in EGCG, are commercially available.

The anticancer effects of green tea and/or its bioactive catechins are well documented in epidemiological, *in vitro* cell culture, *in vivo* animal and human clinical studies; targets for cancer prevention by green tea include cancers of the colon, intestine, liver, lung, ovary, prostate and mammary gland (reviewed in

Singh *et al*., 2011). Many cancer critical molecular targets for tea catechins have been identified, including targets associated with regulation of the cell cycle, apoptosis, cell growth, gene transcription, kinase activity and regulation of the epigenome (Singh *et al*., 2011). By virtue of their antioxidant properties, green tea polyphenols suppress the inflammatory processes that contribute to carcinogenesis, including suppression of TNFα expression and NFκB signaling (Yang *et al*., 1998; Yang *et al*., 2001; Mazzon *et al*., 2005; Byrav *et al*., 2011; Kawaguchi *et al*., 2011), with evidence of modulation of TLRs (Byun *et al*., 2012; Cunha *et al*., 2013). Consumption of green tea polyphenols decreased colonic inflammation, suppressed TNFα expression and reduced markers of oxidative stress in rodents with chemically-induced colitis (Mazzon *et al*., 2005; Oz *et al*., 2005; Oz *et al*., 2013).

Green tea has been studied in different animal models of gastrointestinal cancer with promising results. Supplementation of drinking water with EGCG reduced intestinal tumorigenesis in $Apc^{min/+}$ mice, which are genetically predisposed to the development of small intestinal tumors (Orner *et al*., 2003; Ju *et al*., 2005). The green tea extract polyphenon E has been shown to suppress development of tumors in colons of mice initiated with the carcinogen azoxymethane (AOM) (Ju *et al*., 2003; Ju *et al*., 2007; Shimizu *et al*., 2008), and Xiao *et al*. (2008) showed that green tea polyphenols suppress development of aberrant crypt foci in colons of rats initiated with AOM. Using a mouse model of colon inflammation where mice are provided the inflammatory agent dextran sodium sulfate (DSS), Shirakami *et al.* (2008) showed that supplementation with polyphenon E or EGCG suppressed colon tumor development.

Green tea polyphenols are extensively metabolized by gut microbiota (Schantz *et al*., 2010; Calani *et al*., 2012). Our collaborator recently determined that oral consumption of green tea polyphenols increases abundance of *Bifidobacterium spp*. in mice (Lefevre, personal communication), similar to observations for humans consuming green tea (Jin *et al*., 2012). In a batch culture *in vitro* experiment, Tzounis *et al*. (2008) showed that (+)-catechin incubation increased growth of *Clostridium coccoides*, *Bifidobacterium* spp., and *Escherichia coli*, while attenuating growth of *C. histolyticum*.

5.2 Anthocyanins

Anthocyanin-rich foods (certain red, purple and blue berries and fruits; pigmented grains, nuts and legumes; red and purple vegetables [but not beets]) and derived extracts have long been touted for their health promoting effects pertaining to obesity, diabetes, cardiovascular disease, inflammation and cognitive function (Galli *et al*., 2002; Tsuda, 2012). Dietary supplements containing extracts derived from acai berry, tart cherry, elderberry, blueberry, bilberry, aronia (chokeberry) or black currant are widely available for purchase. The intake of anthocyanins in the U.S. is estimated to be about 12.5 mg/day (Wu *et al*., 2006); however, these compounds are poorly absorbed in the gastrointestinal tract.

Anthocyanidins (the aglycone form of anthocyanins), such as cyanidin and delphinidin, modulate a variety of cell signaling pathways involved in inflammation, carcinogenesis and angiogenesis, including suppression of expression and/or signaling through COX-1 and -2, iNOS, Akt, ERK1/2, TNFα, NFκB, IL-6 and IL-8 (see Domitrovic, 2011; Chen *et al*., 2014). Oral consumption of black raspberry powder (*Rubus occidentalis*), which has high cyanidin content, provided significant protection against chemically-induced colitis via suppression of pro-inflammatory pathways (lower TNFα and IL-1β expression, reduced activity of NFκB and COX-2 in the colon) (Montrose *et al*., 2011). Moreover, dietary supplementation with anthocyanin-rich extracts from tart cherries, pomegranate or purple sweet potato reduced tumorigenesis in the gastrointestinal tract of rodents (Kang *et al*., 2003; Bobe *et al*., 2006; Banerjee *et al*., 2013; Lim *et al*., 2013). Anthocyanins are reported to have anti-microbial activity (Cisowska *et al*., 2011; Miladinovic *et al*., 2014), and the gut microbiome of mice fed anthocyanins from purple corn is very distinct from that fed a standard diet (Lefevre *et al*., 2011). In an *in vitro* fecal batch culture system, Hidalgo *et al*. (2012) reported that a mixture of anthocyanins from grape peel (containing primarily malvidin-, delphinidin- and petunidin-3-glucosides) enhanced growth of *Bifidobacterium* spp. and *Lactobacillus* spp. Finally, evidence from *in vitro*, animal and human volunteer studies shows that anthocyanins are metabolized extensively by the gut microbiome (reviewed in Williamson and Clifford, 2010). Interestingly, Salyer *et al*. (2012) reported that consumption of anthocyanin-rich blackberries conditioned the gut microbiome in mice to more effectively metabolize cyanidin-3-glucoside *in vitro*.

5.3 Proanthocyanidins

Proanthocyanidins are condensed flavan-3-ols and are abundant in cocoa, chocolate, grape seeds and skin, cinnamon, nuts and certain berries (blueberries, choke berries, cranberries). These sources differ with respect to the degree of flavan-3-ol polymerization and type of linkage. Health benefits ascribed to consumption of proanthocyanidin-rich foods and supplements include improvements in insulin sensitivity and inflammation, reduced risk for cancer, cardiovascular disease and urinary tract infections (Ouedraogo *et al*., 2011; Gu and Lambert, 2013; Krueger *et al*., 2013; Yang and Xiao, 2013).

Cocoa powder contains high amounts of flavonoids, including the monomers (–)-epicatechin and catechin and various catechin-based polymers, termed procyanidins (reviewed in Ramiro-Puig and Castell, 2009). Some cocoa-derived products can deliver as much or more polyphenolic antioxidants as other fruit or tea products (Lee *et al*., 2003; Vinson *et al*., 2006). Proanthocyanidins from cocoa (as high as 517 mg/40 g serving of dark chocolate) have been shown to have antioxidant and anti-inflammatory properties *in vitro* (Vinson *et al*., 2006; Rodriguez-Ramiro *et al*., 2011; Rodriguez-Ramiro *et al*., 2012). Most *in vivo* studies on the effects of cocoa polyphenols have employed cocoa powder or a commercial cocoa or chocolate product enriched in polyphenols. For example, dietary supplementation with 0.24% cocoa polyphenols via cocoa powder provided significant protection against colonic inflammation in rats and suppressed activity of NFκB and expression of pro-inflammatory enzymes COX2 and iNOS in the colon (Rodriguez-Ramiro *et al*., 2013). Consumption of a cocoa-rich diet also reduced development of pre-neoplastic lesions in rats initiated with AOM (Rodriguez-Ramiro *et al*., 2011; Hong *et al*., 2013). In healthy human volunteers, consumption of a cocoa drink with high polyphenol content for 4 wk significantly increased *Bifidobacterium spp.* as well as *Lactobacillus* and *Enterococcus spp.*, while abundance of *C. histolyticum* was reduced (Martin *et al*., 2012). Cocoa consumption in rats was also shown to modulate the gut microbiome, leading to a decrease in abundance of members of the *Bacteroides, Clostridium* and *Staphylococcus* genera, but no apparent changes in abundance of *Lactobacilllus* or *Bifidobacterium* (Massot-Cladera *et al*., 2012). Finally, a number of reports have shown that cocoa proanthocyanidins are metabolized by the gut microbiome (Tzounis *et al*., 2008; Fogliano *et al*., 2011).

5.4 Soy isoflavones

Much attention has been given to the apparent link between diet and the lower rate of many cancers in Asian populations compared with US residents, with particular focus on the contribution of soy and soy-based bioactive food components, such as the isoflavone compounds genistein and daidzein (reviewed in Wu *et al*., 2009; Andres *et al*., 2011). Evidence from human and animal studies suggests that consumption of soy-based foods and/or soy isoflavones is associated with reduced risk of risk of several malignancies, including cancers of the mammary gland, ovary, bladder, colon, liver, pancreas, lung, head and neck as well as lymphoma and leukemia (reviewed in Andres *et al*., 2011). Also, others have shown that dietary soy inhibits development of pre-neoplastic lesions in the colon (Zhang *et al*., 2013). Alternatively, there is continued concern that isoflavone consumption is positively associated with risk of endometrial cancer or abnormalities reproductive development based on evidence from animal studies (Santell *et al*., 1997; Newbold *et al*., 2001; Rachon *et al*., 2007).

Genistein and daidzein are well known ligands for the estrogen receptor (ER), and much of their anti-cancer activities are attributed to modulation of ER-dependent cell signaling. Alternatively, genistein has also been shown to modulate the epigenome via inhibition of the activity of DNA methyltransferase, the enzyme responsible for establishing the methylation code and directing expression of many key tumor suppressor genes (reviewed in Zhang and Chen, 2011; Rietjens *et al*., 2013). While much of the cancer prevention research with soy isoflavones has focused on genistein, there is increased interest in the health benefits (or reduced risks) of complex soy mixtures in the form of extracts (Gallo *et al*., 2006) or soy flour (Allred *et al*., 2004; Allred *et al*., 2005).

One of the best characterized examples of a dietary bioactive interacting with the gut microbiome to influence human health is the microbial conversion of soy isoflavones to equol, a non-steroidal estrogenic compound. Many of the cancer protective properties of soy are thought to be derived through the conversion of soy isoflavones to equol, which has been shown to be inversely related to prostate and breast cancer incidence in Asian populations (Lampe, 2010). Production of equol from the soy isoflavone daidzein

requires a gut microbiome with that specific metabolic capacity, yet only about one-third of the population has a resident gut microbiome that can generate equol (Yuan *et al*., 2007). Thus, an individual's microbiome likely influences the potential chemo-preventative properties of dietary soy. Importantly, routine soy consumption appears to impact the composition of the gut microbiome by positively selecting for bacteria that are equol-producers. In countries that traditionally consume soy, such as Japan, China, and Korea, it is estimated that 50 to 60% of the population has a microbiome capable of producing equol (Setchell and Clerici, 2010). In contrast, only 25 to 30% of Westerners can produce equol after consuming isoflavones. Therefore, the potential beneficial effects of soy in prevention of cancer are nuanced and dependent and on an individual's routine diet and gut microbial population.

6. Current challenges and new strategies

6.1 Modeling the typical western diet in preclinical animal studies

The typical Western diet is characterized by inexpensive, highly processed foods that are rich in calories, but low in many essential micronutrients. As most micronutrients are acquired through the diet, consumption of energy-dense, nutrient-poor foods may result in micronutrient intakes below Recommended Daily Allowances (RDAs). RDAs are formulated to prevent deficiency diseases in the U.S. population. However, new evidence suggests that chronic low intakes of micronutrients can negatively affect metabolic processes without triggering the physical manifestation of acute deficiency (reviewed in Ames, 2005). Although these low nutrient intakes do not trigger symptoms of acute deficiency, other adverse health effects from chronic low dietary exposure are possible, including increased risk or acceleration of chronic, degenerative diseases such as cancer, cardiovascular disease and diabetes. While some studies have investigated the health effects of chronic low consumption of single micronutrients (Ames, 2005), information regarding the impact of chronic low intake of multiple micronutrients on disease outcome is lacking, especially in the context of a typical Western diet.

In most studies investigating the contribution of functional foods, bioactive food components and micronutrients for disease prevention (especially cancer), researchers routinely employ standard diets that are generally balanced with respect to macro- and micronutrient levels to optimize rodent health, such as the AIN diets formulated by the American Institute of Nutrition (Reeves *et al*., 1993). In mechanistic studies with model organisms, nonessential nutrients or whole food extracts are often added to these AIN diets to investigate cancer protective effects, or conversely, levels of individual macronutrients or micronutrients are altered to determine their role in carcinogenesis. While this strategy has led to significant findings, our contention is that a rodent diet more representative of the diet consumed by the majority of Americans is necessary to appropriately evaluate colon cancer risk and to develop specific and effective prevention strategies. Some scientists have sought to address this issue by employing "cafeteria" style diets (animals are free to select from a variety of tasty processed foods) in an attempt to emulate typical Western dietary patterns for rodent disease models. However, the cafeteria diet has limited value as an experimental model because it is poorly defined with respect to micronutrient composition and unlikely to provide for robust experimental replication (Moore, 1987; Rothwell and Stock, 1988). Commercial Western diets have also been developed for the study of obesity, namely the DIO diets, which typically contain 45% or 60% of energy as fat and differ from the AIN diets primarily in their high lard and sucrose content (Gajda, 2008). Although these high fat diets effectively induce obesity in rodents (Jawien *et al*., 2004), they are extreme in their sugar and fat compositions when compared to a typical Western dietary pattern and do not differ substantially from AIN diets in micronutrient content (Gajda, 2008). Importantly, none of these approaches for modeling typical Western nutrition has appropriately considered the contribution of suboptimal micronutrient intake in their disease models.

To address this resource gap, our group developed the new total Western diet (TWD) for rodents with energy and nutrient profiles that emulate a typical Western diet using available U.S. survey data (NHANES) (Hintze *et al*., 2012). Briefly, the amount of each macro- and micronutrient in the AIN93G basal diet, a diet routinely used in cancer studies today, was adjusted to match 50th percentile intakes for Americans as reported in NHANES survey data. These mass amounts were then adjusted for caloric intake. The TWD has fewer calories from protein and carbohydrate

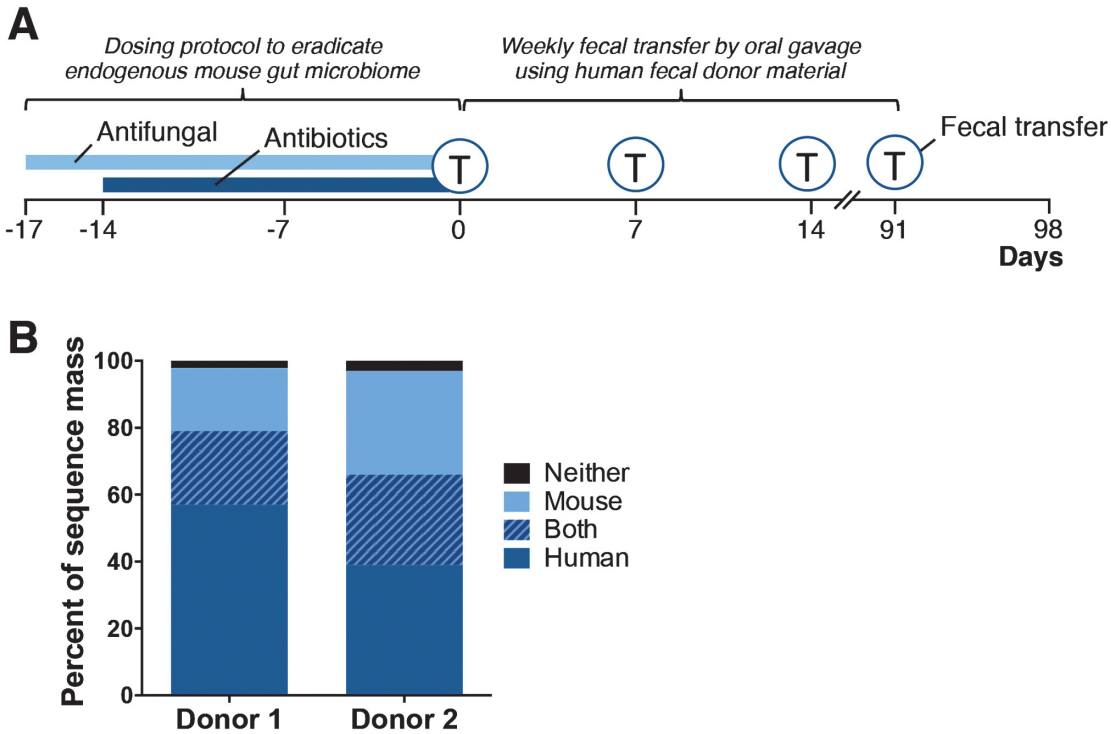


Fig. 7. A) Recommended protocol for establishing a humanized gut microbiome in rodents is shown, including periods of antifungal and antibiotic administration (light and dark green lines) and the timing of oral gavage with donor fecal material (circled T). See Hintze *et al.* (2014) for complete protocol details. B) Chart depicts results following weekly fecal transfer from two human donors to recipient mice using the protocol outlined in panel A. Values shown are the fraction of bacteria sequence mass in recipient mice that originated exclusively from the donor (human), that were shared by the donor and the recipient mouse (both), that were present only in the original mouse microbiome (mouse) or that were not detected in the original human or mouse microbiomes (neither).

sources and twice that from fat as compared to the AIN-93 diet. The new diet contains more saturated and monounsaturated fats, less polyunsaturated fat, more complex carbohydrates and twice the level of simple sugars. TWD includes less calcium, copper, folate, thiamine and vitamins B_6, B_{12}, D and E, but much more sodium. Overall, the TWD is not necessarily extreme in the level of any given nutrient, but rather reflects the overall dietary pattern of the U.S. This newly devised diet that better represents typical U.S. nutrition is highly useful for studies employing animal models of human cancer.

6.2. Modeling the human gut microbiome in preclinical studies

The field of gut microbiology and associated human health outcomes has advanced greatly through the use of "humanized" mouse models (Gootenberg and Turnbaugh, 2011; Turnbaugh *et al.*, 2009; Goodman *et al.*, 2011). Traditionally, these models require seeding germ-free mice with microbiota from human donors, thus providing a useful system to study the interactions between human microbiota and chronic disease in situations where human subjects are not appropriate. However, maintenance of germ-free mouse colonies is expensive and requires substantial institutional investment in infrastructure and specialized personnel. Moreover, germ-free mice are not readily available for the most common and/or most important strains used in health research, including many inbred and genetic mouse models. Thus, to efficiently model the human microbiome in mice, we developed a humanized mouse model using broad-spectrum antibiotics and human fecal transfer (Hintze *et al.*, 2014).

Briefly, the human microbiota fecal transfer method involves the following steps (Fig. 7A): 1) depleting resident animal intestinal microbiota by gavaging the animal twice daily for 17 days with broad spectrum

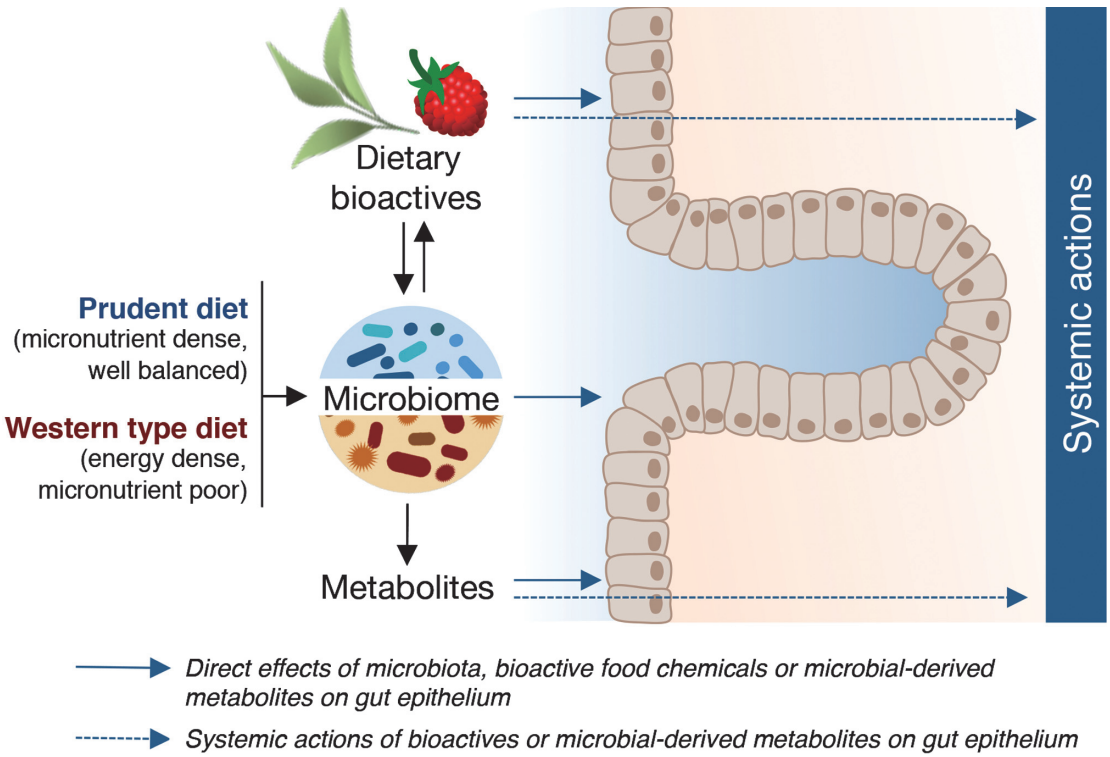


Fig. 8. Proposed model for investigating the impact of dietary bioactives for prevention of colon cancer by incorporating different nutritional patterns (prudent diet versus Western type diet) and human gut microbiota (via new humanized gut microbiome model) as part of the study design.

antibiotics (ampicillin, vancomycin, neomycin and metronidazole) and an antifungal (amphotericin B); 2) introducing human microbiota, derived from frozen fecal samples, by oral gavage weekly; and 3) maintaining the animals in microisolator cages supplied with HEPA filtered air. After 17 days, mice in the fecal transfer treatments were gavaged with fecal material reconstituted in sterile saline from one of two human donors (donor 1 or 2). Mice were gavaged weekly with fecal material from their respective donors until for up to 12 weeks. To assess the effectiveness of this transfer method, bacteria populations of the ceca were characterized by traditional 16S rRNA pyrosequencing. The resulting data were then compared by weighted Unifrac analysis to distinguish differences in the microbiome between treatments. We determined that the microbiome from control mice (no antibiotics), antibiotic-only treated mice and mice receiving either human donor 1 or 2 inoculant were distinct from each other. In mice inoculated with human donor sample, approximately 57 to 68% of the donor sequence mass was recovered in the respective recipient mice (Fig. 7B) (Hintze *et al.*, 2014). Additionally, an analysis of microbial-derived metabolites revealed that the gut microbiomes of mice inoculated with material from donors 1 and 2 were also distinct (Hintze *et al.*, 2014). These data show that our fecal transfer protocol caused substantial changes to the cecal metabolome and that our method is sufficiently robust such that phenotypic differences between mice humanized with microbiota from different human donors are readily apparent. Thus, we expect that humanized mice generated from our protocol can be used for investigations into the contribution of human intestinal bacteria on the etiology of disorders linked to gut microbiota such as colon cancer, inflammatory bowel disease, obesity, diabetes and autism (Kinross *et al.*, 2008; Rowland, 2009; Iebba *et al.*, 2011; Marteau and Chaput, 2011; Musso *et al.*, 2011; Cucchiara *et al.*, 2012; Kootte *et al.*, 2012; Lawrance, 2012; Tehrani *et al.*, 2012).

Although our approach to humanize the gut microbiome of laboratory animals is technologically straightforward, this method has the potential to dramatically impact this field of science. Other investigators who have successfully humanized mouse intestinal microbiota relied on the use of germ-free

mice as recipients and subsequent maintenance of the animals in a dedicated, germ-free vivarium (Turnbaugh *et al*., 2009); however, this approach has substantial (and potentially insurmountable) limitations. Most important of these is the availability of germ-free mice in only a few mouse strains. The vast majority of well-characterized inbred mouse strains and genetically modified mice, all of which are essential models for the study of human disease, are not commercially available as germ-free. This represents a significant limitation to the vast majority of research groups who wish to examine the impact of human microbiota populations in animal models of human disease, but lack the means to derive germ-free animals from the appropriate strain of interest. Also of note, this new approach can be extended to other highly used animal model species (rats, hamsters, etc.). To put it simply, mice of any strain or genetic model can have their intestinal microbiota humanized on demand as needed by the investigator following this protocol for human microbiota transfer to rodents.

7. Conclusions

While abundant evidence from pre-clinical studies supports the strategy of diet modification to reduce cancer risk, there still exist many knowledge gaps on the role of dietary bioactives for modification of the gut microbiome to influence disease development. The role of gut bacteria in maintaining health and the impact of dysbiosis of the microbiota ecosystem in triggering or exacerbating disease is widely recognized (e.g., Round and Mazmanian, 2009; Guinane and Cotter, 2013; Schwabe and Jobin, 2013; Festi *et al*., 2014; Giannelli *et al*., 2014; Sanz *et al*., 2014; Schippa and Conte, 2014; Tojo *et al*., 2014; Lei *et al*., 2015; McLean *et al*., 2015). Yet, the impact of the gut microbiome on the efficacy of many dietary bioactives for preventing cancer has been relatively overlooked. Moreover, given the observations that basal diet can markedly influence the composition of the gut microbiome and that different gut microbiota populations confer different metabolic activities towards dietary bioactives, it is critically important to consider the impact of both basal diet and the gut microbiome in animal studies investigating dietary bioactives as chemopreventive agents. Thus, we propose an integrated, more translational methodological approach for such studies (Fig. 8) that incorporates the Western type diet (macro- and micronutrient composition) as part of the experiment design and utilizes a humanized gut microbiome to address the role of gut bacteria in health maintenance and/or disease development. The development of the new total Western diet and a straightforward protocol for human microbiota fecal transfer support this new experimental model.

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