

# The intestinal microbiota, gastrointestinal environment and colorectal cancer: a putative role for probiotics in prevention of colorectal cancer?

M. Andrea Azcárate-Peril,<sup>1</sup> Michael Sikes,<sup>2</sup> and José M. Bruno-Bárcena<sup>2</sup>

<sup>1</sup>Department of Cell and Molecular Physiology, University of North Carolina School of Medicine, Chapel Hill;

<sup>2</sup>Department of Microbiology, North Carolina State University, Raleigh, North Carolina

Submitted 21 March 2011; accepted in final form 20 June 2011

**Azcárate-Peril MA, Sikes M, Bruno-Bárcena JM.** The intestinal microbiota, gastrointestinal environment and colorectal cancer: a putative role for probiotics in prevention of colorectal cancer? *Am J Physiol Gastrointest Liver Physiol* 301: G401–G424, 2011. First published June 23, 2011; doi:10.1152/ajpgi.00110.2011.—Colorectal cancer (CRC) is the third most commonly diagnosed cancer in the United States, and, even though 5–15% of the total CRC cases can be attributed to individual genetic predisposition, environmental factors could be considered major factors in susceptibility to CRC. Lifestyle factors increasing the risks of CRC include elevated body mass index, obesity, and reduced physical activity. Additionally, a number of dietary elements have been associated with higher or lower incidence of CRC. In this context, it has been suggested that diets high in fruit and low in meat might have a protective effect, reducing the incidence of colorectal adenomas by modulating the composition of the normal nonpathogenic commensal microbiota. In addition, it has been demonstrated that changes in abundance of taxonomic groups have a profound impact on the gastrointestinal physiology, and an increasing number of studies are proposing that the microbiota mediates the generation of dietary factors triggering colon cancer. High-throughput sequencing and molecular taxonomic technologies are rapidly filling the knowledge gaps left by conventional microbiology techniques to obtain a comprehensive catalog of the human intestinal microbiota and their associated metabolic repertoire. The information provided by these studies will be essential to identify agents capable of modulating the massive amount of gut bacteria in safe noninvasive manners to prevent CRC. Probiotics, defined as “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host” (219), are capable of transient modulation of the microbiota, and their beneficial effects include reinforcement of the natural defense mechanisms and protection against gastrointestinal disorders. Probiotics have been successfully used to manage infant diarrhea, food allergies, and inflammatory bowel disease; hence, the purpose of this review was to examine probiotic metabolic activities that may have an effect on the prevention of CRC by scavenging toxic compounds or preventing their generation *in situ*. Additionally, a brief consideration is given to safety evaluation and production methods in the context of probiotics efficacy.

lactic acid bacteria

ACCORDING TO THE CENTERS FOR DISEASE CONTROL AND PREVENTION (CDC), colorectal cancer (CRC) is the third most commonly diagnosed cancer in the United States. In 2005, more than 141,000 Americans (72,007 men and 69,398 women) were diagnosed with CRC. Men and women had significantly different incidence rates. Black men had the highest rates (64.9/100,000), white men were second (55.4/100,000), followed by Hispanic men (46.8), Asian/Pacific Islander men (40.6), and American Indian/Alaska Native men (34.3). Among women, black women were the most likely to be diagnosed with colorectal cancer in 2005 (49.4/100,000) followed by white

women (40.8), Hispanic women (33.9), Asian/Pacific Islander women (32.2), and American Indian/Alaska Native (24.5) (51). In the European Union, CRC is the second most common cancer and second major cause of death in men and women (35).

The present review summarizes the advances in the characterization of the human gastrointestinal (GI) microbiota, how the microbiome might contribute to the development of CRC and, on the basis of this information, how “tailored probiotics” might be used to modulate specific bacterial populations of the GI tract to restore an equilibrated microbiota and intestinal microenvironment, which can contribute to CRC prevention.

## *Colorectal Cancer: Genetics, Diet, and Lifestyle*

Five to fifteen percent of all CRC cases can be attributed to the following hereditary CRC syndromes: Lynch syndrome

Address for reprint requests and other correspondence: M. A. Azcárate-Peril, Dept. of Cell and Molecular Physiology, 312 Isaac Taylor Hall - Campus Box 7545, School of Medicine, Univ. of North Carolina, Chapel Hill, NC 27599-7545 (e-mail: azcarate@med.unc.edu).

(also hereditary nonpolyposis CRC or HNPCC), familial adenomatous polyposis (FAP), and MUTYH-associated polyposis (MAP) (50). Lynch syndrome is an autosomal-dominant disease characterized by early-onset CRC and the presence of multiple neoplasms affecting the colon, rectum, and other organs (263). HNPCC is associated with germline mutations in the mismatch repair (MMR genes), mainly MSH2, MLH1, MSH6, and PMS2 (65, 94). FAP is characterized by the early onset of hundreds to thousands of adenomas throughout the large bowel, attributable to a germline mutation in the adenomatous polyposis coli (APC) gene, located on chromosome 5q21. APC is a tumor suppressor gene, first localized in 1987 and cloned in 1991 following mutation analyses in unrelated families with FAP (reviewed in Ref. 71). MAP is an autosomal recessive disorder characterized by adenomatous polyps of the colorectum and a very high risk of colorectal cancer (187).

CRC is thought to develop over many years in a multistep process, known as the “adenoma-carcinoma sequence” (273). Early studies showed the involvement of APC in FAP (113). Subsequent studies revealed that mutations in APC are also found in 63% of sporadic adenomas and up to 80% of sporadic colorectal cancers (146, 179, 208). APC is a multidomain protein that contains binding sites for numerous proteins, including microtubules, the Wnt/Wg pathway components  $\beta$ -catenin and axin, the cytoskeletal regulators EB1 and IQGAP1, and the Rac guanine-nucleotide-exchange factor Asef1 (11). APC acts as a “gatekeeper gene”, maintaining low levels of  $\beta$ -catenin in the absence of a Wnt signal, thus preventing excessive cell proliferation. On the other hand, HNPCC has been used to identify an alternative pathogenesis mechanism that involves “caretaker” MMR genes responsible for recognizing and repairing single-base and larger-strand slippage mismatches in DNA replication (65).

Although there is a strong genetic component in the development of colorectal adenomas or CRCs, it is generally accepted that environmental factors including diet and lifestyle have a major impact on risk. Lifestyle aspects

related to increased risk of CRC include elevated body mass index (BMI), obesity, and low physical activity (128, 165). A large number of dietary compounds have been associated with either increased or decreased risk of colon cancer [see Fig. 1, based on reviews or papers by Marshall (165); Butt and Sultan (43); Kim and Kwon (133); Berquin et al. (26); Pool-Zobel and Sauer (207); and Johnson and Lund (128)]. Broadly, elevated risk of CRC has been associated with high consumption of red and processed meat, refined grains, sweets, and alcohol, and to a low consumption of fruits and vegetables (19, 215).

*Metabolic Signatures of the GI Environment*

The intestinal environment including the gut luminal content is defined by the colliding processes of nutrient absorption, host defense, and host-microbial interactions. The GI milieu is highly variable across individuals because of inherent inter-individual variation and environmental factors including diet and lifestyle. High-resolution magic-angle spinning (HRMAS) <sup>1</sup>H NMR spectroscopy in combination with orthogonal projection to latent structure-discriminant analysis (O-PLS-DA) was recently used to investigate metabolic profiles of human intestinal tissue samples (276). The typical <sup>1</sup>H Carr-Purcell-Meiboom-Gill HRMAS NMR spectra of intact human biopsies of antrum, duodenum, jejunum, ileum, and transverse colon showed 35 common metabolites including amino acids, carboxylic acids, pyrimidines, membrane component metabolites, creatine, glucose, inositols, lipids, and triglycerides. These metabolites were found in the various human GI tract samples irrespective of the sampling position. However, variation in the relative signal intensities indicated different concentrations of components in different intestinal locations. O-PLS-DA of NMR spectra of samples indicated that 1) there was no sex variation in any part of the GI tract mucosa; 2) variations between duodenum and jejunum were negligible; 3) the antrum

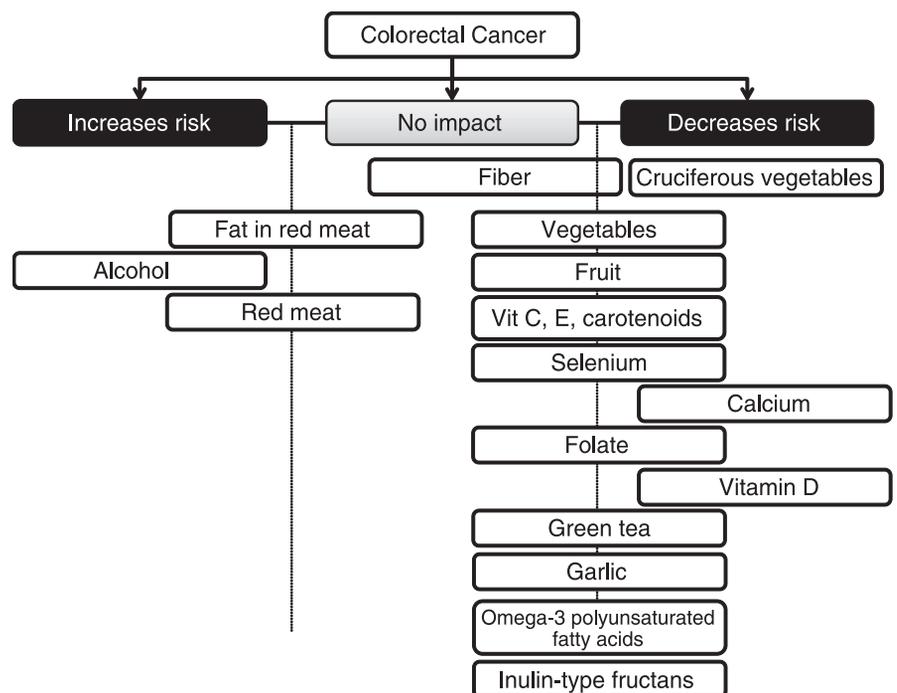


Fig. 1. Dietary compounds associated with either increased or decreased risk of colorectal cancer [Based on reviews or papers by Marshall (165), Butt and Sultan (43), Kim and Kwon (133), Berquin et al. (26), Pool-Zobel and Sauer (207), and Johnson and Lund (128)].

contained higher levels of choline, glycogen, phosphorylethanolamine, taurine, and acetyl glycoproteins, and lower levels of amino acids, lipids, glucose, and lactate compared with other gut regions; 4) the duodenum and jejunum were similar in terms of their biochemical composition and were rich in choline, glutathione, glycerophosphocholine (GPC), lipids, and glycoproteins but contained relatively lower levels of acetate, betaine, glycogen, inositols, and phosphorylethanolamine; 5) the ileum was rich in amino acids, but low in GPC; and 6) the transverse colonic mucosa contained higher levels of acetate, glutamate, inositols, lactate, with lower levels of creatine, GPC, and taurine (Fig. 2) (276).

A similar global biochemical composition of the different GI tract regions was reported for conventional and conventionalized mice in a study by Martin et al. (166). This study aimed to investigate the impact of the intestinal microbiota on the biochemical composition of intact tissues obtained from conventional mice, conventionalized mice, and mice transplanted with a simplified human baby microbiota supplemented with probiotics and supplemented with synbiotics.

#### Modification of the GI Environment by CRC

Research studies have shown that the tumor microenvironment, the environment within colorectal neoplastic lesions, is significantly different to the normal intestine. The modified tumor environment can have adverse effects on drug uptake and the intrinsic sensitivity of malignant cells to treatment (175, 176). Early  $^1\text{H}$  NMR spectroscopy studies of colon tumors vs. normal mucosal biopsies demonstrated a significant increase in the concentration of the endogenous compounds lactate, glutamate, aspartate, taurine, spermine, glutathione and glycerophosphoethanolamine, and a significant decrease of myo- and scyllo-inositol (183, 184). A recent quantitative

metabolome profiling of the colon cancer microenvironment by Hirayama et al. (114) confirmed increased concentrations of most amino acids in colon tumors with the exception of glutamine. Additionally, decreased levels of glucose were detected in tumor tissue, which can be explained by the fact that increased glycolytic activities in cancer cells deplete glucose in a hypovascular microenvironment. This fact also explains an increased concentration of lactate and a decreased concentration of pyruvate in tumor tissue. This study also identified higher levels of the tricarboxylic acid (TCA) metabolites succinate, fumarate, and malate in tumor samples, which contradicts the studies by Chan et al. (53) and Denkert et al. (76). Both studies reported lower levels of metabolites of the TCA cycle, which confirm previous proteomic reports that show an impaired TCA cycle in colon carcinoma tissues (6, 27). Chan et al. (53), using HR-MAS NMR, identified 10 marker metabolites that separated normal from tumor samples. Lipids, polyethylene glycol, and glucose were identified at higher levels in normal mucosa compared with tumor tissues, whereas choline-containing compounds, taurine, scyllo-inositol, glycine, phosphoethanolamine, lactate, and phosphocholine were present at higher levels in the CRC samples. Twenty-four additional metabolites were identified in the same study by gas chromatography mass spectrometry, of which only fumarate, malate, mannose, galactose, glucose, 1-hexadecanol, and arachidonic acid showed increased levels in normal mucosa compared with tumor samples. Increased concentrations of lactate, phosphate, L-glycine, L-proline, L-phenylalanine, palmitic acid, marganic acid, oleic acid, stearic acid, uridine, 11,14-eicosadienoic acid, 11-eicosenoic acid, 1-O-heptadecylglycerol, 1-monooleoylglycerol, propyl octadecanoate, and cholesterol were found in tumor samples (53).

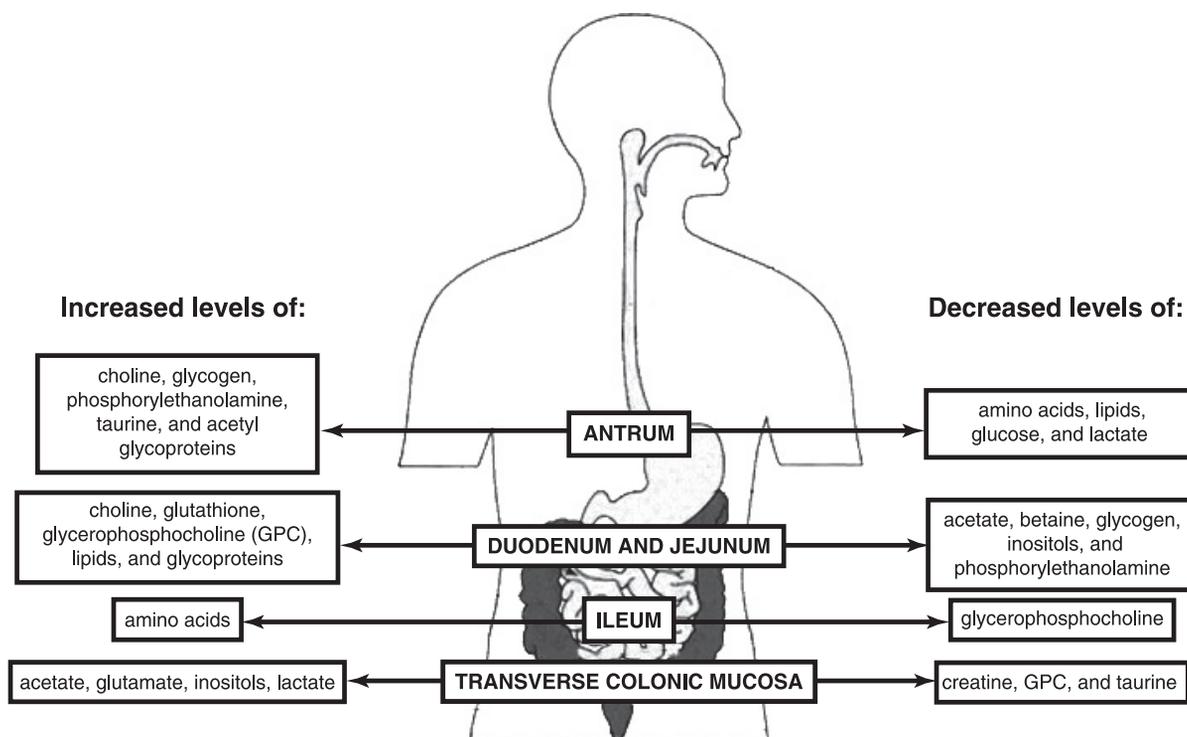


Fig. 2. Biochemical composition of the different gastrointestinal regions [Adapted from Wang et al. (276)].

### Immune Surveillance of the GI Tract

Because humans and their microbiota have coevolved, the human immune system is designed to generally tolerate commensals while retaining the capacity to rapidly mobilize against invading pathogens. Not surprisingly, the gut immune system is distinct in both form and function from other components of human immunity, with the extensive involvement of normal microbiota in maintaining an effective epithelial barrier and with lymphocyte induction occurring at sites disseminated throughout the GI lamina propria (95). The barrier capacity of the mucosal epithelium derives from an extensive network of tight junctions (TJ) that stitch the epithelial cells together. Pathogenic disruption of TJs can induce inflammation, which then further exacerbates TJ leakiness, as seen when enteropathogenic *Escherichia coli* (*E. coli*) triggers dephosphorylation and disassociation of the TJ protein, occludin (247). Toll-like receptor (TLR)-dependent activation of epithelial cells, macrophages, and dendritic cells (DCs) proximal to the pathogenic lesion initiates an inflammatory response characterized by release of proinflammatory cytokines including IL-1 $\beta$ , IL-6, IL-8, IL-12, IL-13, IL-17, IL-23, IFN- $\gamma$  and TNF- $\alpha$  that exert wide-ranging effects on the mucosa [for example, IL-13 along with the chemokine CXCL10 induces epithelial cell cycling (61)] and recruit immune effector cells. Inflammatory signals are balanced by tolerogenic cytokines, particularly IL-10 produced by T<sub>Regs</sub> to promote oral tolerance of food compounds and normal bacterial microbiota.

A robust gut commensal microbiota can provide an effective barrier against pathogenic invasion. The gut epithelial cells of mice raised in a germ-free environment show reduced levels of ATP, major histocompatibility complex II, and TLR-9 and are attached to a lamina propria that is thinner and less cellular than in normally colonized mice (reviewed in Ref. 228). At the same time, the gut microbiota guides both development and function of the gut immune system. Before colonization, inductive sites are reduced in both number and size in the guts of germ-free animals (33). Likewise, dimeric IgA production and effector T cell numbers are reduced in the small intestine of germ-free animals (122, 125, 182). Select commensals including species of *Bifidobacterium*, *Bacteroides*, and *Lactobacillus* have been linked to various immune-restoring activities including development of inductive tissue, restoration of numbers, and balance between various T<sub>H</sub> and T<sub>Reg</sub> subsets (170) and epithelial immune functions (2, 49). At the same time, commensals exert proinflammatory signals including activation of TLR9 (109, 125) and IL-17 (125).

Chronic inflammation has been linked to CRC (68, 84, 135, 260). In pathological conditions like inflammatory bowel disease (IBD), homeostasis gives way to a chronic inflammatory state characterized by massive immune infiltration, immune-mediated tissue destruction, and attendant disruption of epithelial function and morphology. Gut bacteria such as *Bacteroides fragilis* and *Streptococcus bovis* have been linked to CRC because of their ability to activate immune cells to release promotogenic and proangiogenic cytokines like IL-6 and IL-17 (85, 283). Consequently, IBD conditions including ulcerative colitis and Crohn's disease dramatically increase a patient's risk of developing CRC. Indeed, epidemiological data suggest that up to 15% of human cancer incidence is associated with inflammation (142, 161).

Evidence suggests that IBD develops at least in part as a response to changes in the normal microbiota (dysbiosis) rather than from pathogenic invasion (170, 203, 234). For example, the abundance of *Faecalibacterium prausnitzii*, which has been shown to increase anti-inflammatory IL-10 and reduce proinflammatory TNF- $\alpha$  levels in the mouse colon following oral administration, is reduced in a significant percentage of patients suffering from Crohn's disease (251). The dysbiosis model of IBD proposes that genetic or environmental changes alter gut homeostasis and shift the microbial balance away from symbiotic species (those with known health-promoting effects) and toward pathobiotic species (organisms with pathogenic potential such as *Clostridium* and *Helicobacter* that are resident but not normally pathogenic) (228). This shift in turn leads to the induction of an inflammatory state that becomes chronic and significantly increases the risk for CRC.

A growing body of evidence suggests that probiotics can alter both the basal and induced states of gut immunity. Lactic acid bacteria (LAB) in particular have been shown to induce DC maturation and subsequent T<sub>Reg</sub> activation in the gut (83) and also to induce natural killer cell cytotoxicity and cytokine secretion in peripheral blood mononuclear cells (74). Additionally, *Lactobacillus sobrius* (*L. sobrius*) has been shown to stabilize epithelial TJs in the gut, counteracting the occluding depletion caused by *E. coli* K88 (5). Activation of mesenteric lymph node T<sub>Reg</sub> upon ovalbumin injection correlated with elevated production of IL-10 and transforming growth factor- $\beta$  when rats were pretreated with *L. rhamnosus* GG (227). LAB treatment of the gut epithelial cell line, Caco-2, induces production of the human  $\beta$ -defensin-2 defensin and inhibits LPS-induced IL-23 secretion via TLR-2-dependent signaling (200). Finally, consistent with their anti-inflammatory effect on cultured epithelial cells, LAB strains including *L. casei*, *L. delbrueckii*, and *L. acidophilus* fed to Balb/c mice enhanced production of IgG1 and IL-4, indicative of a Th2-dominated immune response to injected ovalbumin (202).

Although probiotic bacterial interactions with the gut (and systemic) immune system are extremely complex, the data clearly suggest a modulatory effect on gut inflammation, with LAB particularly stabilizing epithelial TJs, inducing epithelial defensin production, and inducing the anti-inflammatory and immunomodulatory capacity of T<sub>Reg</sub> and their DCs. Much work remains to determine how individual modulations impact overall gut health and development, which probiotics exert particular effects, and how probiotics can best be used to shape the gut immune state.

### The Normal Intestinal Microbiota and the Microbiota Associated with CRC

Nonpathogenic commensal microbiota have a profound impact on normal GI physiology. They ensure effective intestinal mucosal motility, growth, and immunity as well as nutrient digestion, absorption, angiogenesis, and fortification of the mucosal barrier. Additionally, bacteria promote host epithelial cell production of fucosylated glycans (on which many gut bacteria feed) (228). Other functions of the GI microbiota include energy recovery from poorly digestible nutrients, modification of bile acids (BAs), and the nutritional supplementation of additional auxotrophic compounds not obtained through diet such as folate and biotin (129, 268).

The majority of the bacteria comprising the microbiota are uncultivable, which meant in the past that researchers were unable to characterize them. Since Antony van Leeuwenhoek observed and described what he called “living animalcules”, traditional microbiology has focused on the study of individual species as isolated units. However, many species have never been successfully isolated as viable specimens for analysis, presumably because we have not been capable of reproducing their optimal growth conditions experimentally. High-throughput sequencing and molecular taxonomic technologies are filling the gaps left by conventional microbiology techniques to provide a more comprehensive catalog of the normal microbiota. Phylogenetic analysis of bacterial 16S rRNA genes, amplified directly from complex communities, identified 82 distinct operational taxonomic units in human fecal samples with the vast majority (95%) distributed among three major monophyletic groups: the *Bacteroides* group, the *Clostridium* coccooides group, and the *Clostridium leptum* subgroup (253). Another comprehensive culture-independent phylogenetic analysis of microorganisms from 107 samples of resected tissue from Crohn’s disease, ulcerative colitis, and control patients without patients showed that, regardless of disease state or anatomical site of sampling, the majority of sequences were associated

with the same four phyla of the bacteria: *Firmicutes* (49% of clones), *Bacteroidetes* (23%), *Proteobacteria* (21%), and *Actinobacteria* (5%), with almost half of the sequences (45%) belonging to two subgroups, the order *Bacteroidales* and the family *Lachnospiraceae* (which comprises the *Clostridium* XIVa and IV groups within the order *Clostridiales*) (89). Comparison of small-subunit rRNA sequences from non-IBD colon and small intestine samples indicated differences in microbial populations with respect to the sampling site (Fig. 3). The colon contained more *Bacteroidetes* and more *Actinobacteria* species compared with the small intestine, which contained more *Firmicutes*. Within this phylum, the small intestine was enriched in species of the family *Streptococcaceae*, and a higher relative percentage of species of *Lactobacillaceae* was observed in the colon (89). The most recent data generated by deep sequencing of the human intestinal microbiota has revealed that most bacterial species are present at low abundance (species defined as organisms sharing  $\geq 97\%$  sequence identity in their 16S rRNA genes) (212). Qin et al. (212) concluded that humans possess  $\sim 1,000$  different bacterial species in the GI tract, close to the 878 and 768 species detected in genetically identical twins by Turnbaugh et al. (269). Although most authors agree on a human intestinal core microbiota at the

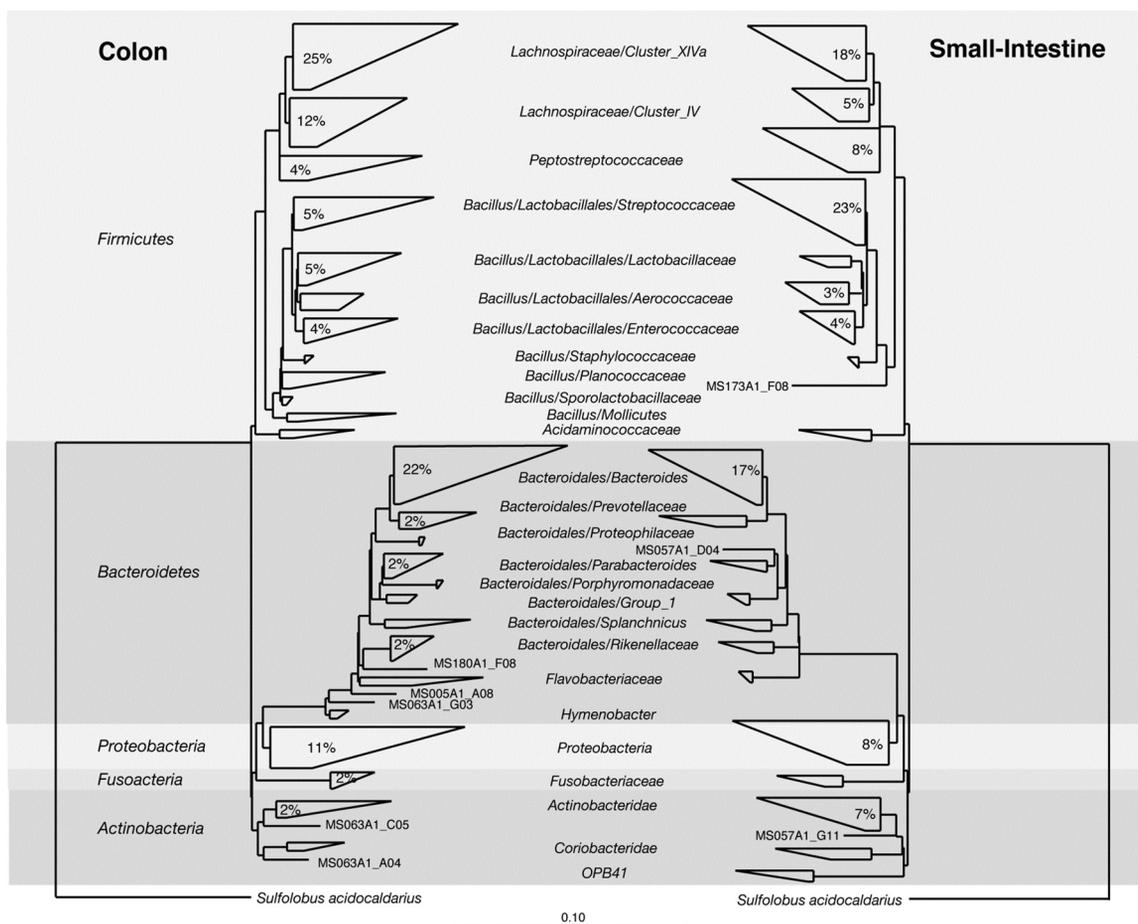


Fig. 3. Comparison of SSU rRNA sequences isolated from colon and small intestine specimens. The phylogenetic trees depict classification of sequences isolated from non-inflammatory bowel disease (IBD) colon (left) and non-IBD small intestine (right) samples. Numbers within wedges represent the proportion of sequences within each sample set (i.e., colon or small intestine) that were assigned to a particular clade (values  $< 2\%$  are omitted). Wedge widths represent the taxa with the longest (top) and shortest (bottom) distances within the clade. Wedge areas represent the number of taxa in each clade. The scale bar represents base changes per site. [Reproduced with permission from Frank et al. (89)].

phylum level composed of *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Actinobacteria* (59, 60, 212, 258, 266), the presence of a core microbiota at the genus or species level is still under debate. Qin et al. (212) identified a common core with 75 species common to >50% of individuals and 57 species common to >90%, whereas Turnbaugh et al. (269) concluded that only 35.9% and 49.1% of the species-level phylotypes found in the fecal communities of the two twins were shared between the two fecal samples, indicating the absence of a core set of abundantly represented universally shared phylotypes. The analysis by Claesson et al. (60) revealed a core gut microbiota across four samples (4 elderly subjects ages 60–87 yr) that consisted of species from *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Actinobacteria*, and at the genus level it consisted of *Alistipes*, *Anaerostipes*, *Anaerotruncus*, *Anaerostipes*, *Anaerotruncus*, *Bacteroides*, *Bifidobacterium*, *Blautia*, *Clostridium*, *Coprococcus*, *Dorea*, *Eubacterium*, *Faecalibacterium*, *Holdemania*, *Leuconostoc*, *Oscillospira*, *Peptostreptococcus*, *Roseburia*, *Ruminococcus*, and *Streptococcus*. The study by Qin et al. (212) added the following genera to the previous list: *Collinsella*, *Parabacteroides*, *Akkermansia*, *Prevotella*, *Butyrivibrio*, *Escherichia*, *Gordonibacter*, *Mollicutes*, and *Bryantella*.

A number of research studies have catalogued the composition of the gut microbiome of healthy humans (212, 267) and the microbiome associated with IBD (105, 156, 280) and obesity (147, 265, 266); however, characterization of the CRC-associated gut microbiota is still behind. Recent deep-sequencing analysis (250) conducted on 12 stool samples (6 with CRC and 6 with normal colonoscopy) selected from 179 patients revealed a total number of 819 genera distributed among cancerous and normal samples with few variations between replicates. A significant difference between CRC and normal samples was observed for the *Bacteroides/Prevotella* group, with higher bacterial counts in cancer samples. Interestingly, the levels of *Bacteroides/Prevotella* were not influenced by age, BMI, family history, or size and location of tumors. In contrast, another study showed that the composition of the mucosa-associated microbiota differed significantly between colon adenoma cases and controls, with increased abundance of *Proteobacteria* and decreased levels of *Bacteroidetes* in adenoma samples (245). This study also demonstrated a higher overall diversity in adenoma samples, which was previously shown for two specific *Clostridium* species in an earlier study conducted in fecal samples of patients with CRC (236). A culture-dependent study of the microbiota of patients with polyps and CRC identified 15 bacterial taxa significantly associated with high risk of colon cancer (including species of *Bacteroides*, *Eubacterium*, and *Bifidobacterium*) and five associated with low risk of colon cancer (including species of *Lactobacillus*) in Japanese-Hawaiians, North American Caucasians, and rural native Japanese populations (181).

#### Biological Activities of the Intestinal Microbiota That Contribute to CRC

Factors involved in the modulation of the intestinal epithelial cell function present in the lumen may include dietary compounds, products of alimentary secretions from salivary glands, the stomach, pancreas, or intestinal glandular cells, secreted regulatory peptides, and constituents or products of the micro-

biota (77). It has been proposed that a steady state exists, where the end products of carbohydrate metabolism in the intestine (butyrate, acetate, and propionate) have a beneficial effect because they can act as colonic nutrients, whereas the products of the metabolism of proteins (phenolic compounds, amines, ammonia, *N*-nitroso compounds, and indoles) might have detrimental effects on the host (69). Although extreme, this concept is interesting because it provides a basis for the study of beneficial vs. detrimental bacteria and their metabolites.

The intestinal microbiota has been linked to CRC by production of toxic and genotoxic bacterial metabolites that can lead to mutations by binding specific cell surface receptors and affecting intracellular signal transduction. Using germ-free and gnotobiotic technology, Uronis and collaborators (270) recently showed that the intestinal microbiota has a direct impact on the development of colitis-associated colon cancer. In this study, azoxymethane-treated *Il10*<sup>-/-</sup> mice develop colitis-associated colon cancer in the presence of *Bacteroides vulgatus*, whereas germ-free mice remain disease free. The presence of colitis directly correlates with tumor multiplicity and acts as a promoter of CRC. Additionally, disruption of MyD88 signaling, a key integrator of multiple Toll-like receptors, prevented the development of colorectal tumors in *Il10*<sup>-/-</sup> mice, indicating a role of the microbiota in triggering intestinal inflammation and neoplastic changes in a susceptible host. The following subsections present bacterial activities presumed to generate metabolites involved in colorectal carcinogenesis. Table 3 lists the probiotic features of potential importance in CRC prevention and the bacterial activities previously linked to increased risk of CRC.

**Secondary bile salt transformations.** An interesting study by O'Keefe et al. (192) highlighted that colon cancer is extremely rare in native African populations (less than 1/100,000 compared with 65/100,000 in African Americans) and correlated the higher incidence of colon cancer in African Americans with a diet high in meat and animal fat and with higher counts of 7 $\alpha$ -dehydroxylating colonic bacteria. Bacterial biotransformations of conjugated BAs begin with bile salt hydrolases (BSH) that deconjugate liver-derived conjugated BAs to liberate primary BA, cholic acid (CA), chenodeoxycholic acid (CDA), and amino acids, which are further modified through dehydroxylation, dehydrogenation, and sulfation. On the basis of the composition of BA in feces of healthy individuals, 7 $\alpha$ -dehydroxylation is quantitatively the most important secondary bacterial bile salt transformation in the human large intestine, yielding deoxycholic acid (DCA) and lithocholic acid (LCA) (221). High concentrations of secondary BAs in feces, blood, and bile have been linked to cholesterol gallstone disease and colon cancer (173). There is considerable experimental evidence that secondary BAs, such as DCA, are cytotoxic to colonic epithelial cells, as well as mutagenic with antiapoptotic properties (25). DCA and other hydrophobic BAs increase oxidative stress through generation of reactive oxygen species (ROS), which in turn cause oxidative DNA damage, in part, through the iron-catalyzed Fenton reaction (31), and impair the human mismatch repair system (54). Figure 4 shows a schematic to illustrate potential roles of BA in DNA damage and GI cancer (25).

BSHs are widely distributed in the genera *Lactobacillus* and *Bifidobacterium*, members of the lactic acid bacteria, most of which are considered probiotics (23). In contrast, 7 $\alpha$ -dehy-

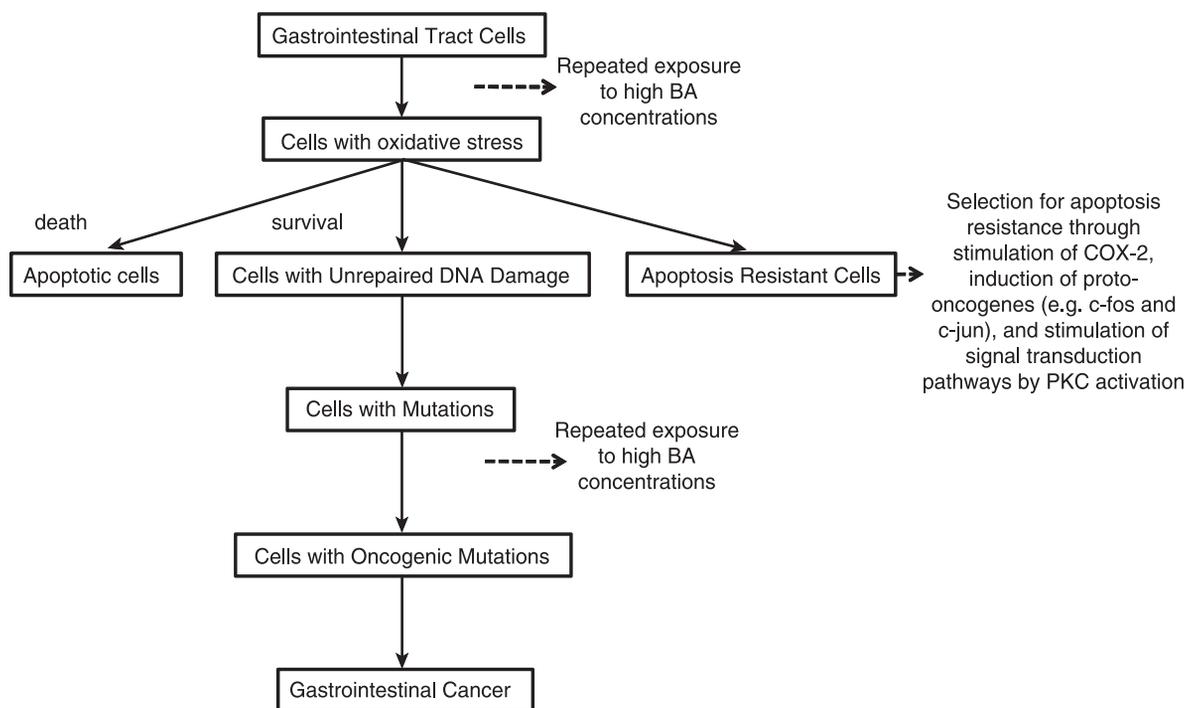


Fig. 4. Pathway of induction of DNA damage and gastrointestinal cancer by bile acid (BA). COX-2, cyclooxygenase-2. [Modified from Bernstein et al. (25)].

droxylation activity is prevalent in species of the genus *Clostridium*, including *C. absonum*, *C. scindens*, *C. bif fermentans*, *C. limosum*, and *C. hylemonae* (221).

**Production of hydrogen sulfide.** A diet high in meat has been shown to significantly increase the levels of taurine conjugation to bile acids. Unlike glycine, taurine contains a sulfonic acid moiety that is reduced and dissimilated to hydrogen sulfide after deconjugation (221). Meat is a rich source of dietary sulfur (through sulfur-amino acids), which promotes the growth of sulfur-reducing bacteria (SRB). SRB have the ability to use sulfate ( $\text{SO}_4^{2-}$ ) as an oxidant to degrade organic matter. An equivalent amount of hydrogen sulfide ( $\text{H}_2\text{S}$ ) is formed per mole of sulfate reduced (120, 191). Hydrogen sulfide is a toxic compound that generates free radicals (18), impairs cytochrome oxidase, suppresses butyrate utilization, and inhibits the synthesis of mucus and the methylation of DNA (57). In fact, a study by Ramasamy et al. (214) concluded that ineffective detoxification of hydrogen sulfide by dysregulation of the two rhodanese isoenzymes thiosulfate sulfurtransferase and mercaptopyruvate sulfurtransferase may result in mucosal insult, inflammation, and ultimately CRC. The diversity and ecology of human colonic SRB is largely unknown although one study identified the main SRB as lactate- and  $\text{H}_2$ -utilizing *Desulfovibrio* spp. (64–81%), acetate-utilizing *Desulfobacter* spp. (9–16%), propionate- and  $\text{H}_2$ -utilizing *Desulfobulbus* spp. (5–8%), lactate-utilizing *Desulfomonas* spp. (3–10%), and acetate- and butyrate-utilizing *Desulfotomaculum* spp. (2%) (97).

**Production of aglycones.** Glycoside hydrolases (glucosidases, EC 3.2.1.-) are a widespread group of enzymes that hydrolyze the glycosidic bond between two or more carbohydrates or between a carbohydrate and a noncarbohydrate moiety. They remove one monosaccharide from the nonreducing end of their substrates in each catalytic cycle with release of

aglycones (162). McBain and Macfarlane (172) showed that most of the glucosidase activities were associated with the bacterial fraction of colon contents. Older reports showed that cycasin (methylazoxymethanol- $\beta$ -D-glucoside), a toxic aglycone released from the nuts, roots, and leaves of *Cycas circinalis* and *Cycas revoluta* by the action of  $\beta$ -glucosidases present in the plant or produced by the intestinal microbiota, was carcinogenic in rodents and has hepatotoxic, neurotoxic, teratogenic, and radiomimetic (imitates the effects of radiation) properties (115).

**Bacterial  $\beta$ -glucuronidases.** The colonic microbial community may convert innocuous compounds into carcinogenic metabolites through a number of enzymatic activities, of which  $\beta$ -glucuronidation has been the most extensively investigated as a biomarker of CRC risk. A number of compounds, including carcinogens formed in food during cooking, drugs, etc., are metabolized in the liver, conjugated to glucuronic acid, and excreted into the small intestine via the bile duct (118, 229, 252). Once these components reach the intestinal tract, they act as substrates for host and bacterial enzymes. Specifically, the  $\beta$ -glucuronidase activity in the GI tract is influenced by diet and composition of the microbiota because only some of the members of the microbiota possess this enzyme. A study of 40 intestinal strains of the phylogenetic groups *Clostridium* (clusters IV, XIVa, and XVI), *Bifidobacterium* sp., and *Bacteroides* sp. showed that ~30% of the clostridia but none of the *Bacteroides* and *Bifidobacterium* species had  $\beta$ -glucuronidase activity (66). Additionally, fecal specimens of patients with colon cancer were reported to have significantly higher  $\beta$ -glucuronidase activity compared with healthy subjects (132).

With regard to diet, the carcinogenic properties of 2-amino-3-methylimidazo(4,5-f)quinoline (IQ), which is one of the heterocyclic amines formed when various meats and fish are cooked, have been directly correlated with the presence of the

bacterial  $\beta$ -glucuronidase encoded by the *uidA* gene in *E. coli*. In gnotobiotic rats monoassociated with a wild-type strain of *E. coli* (carrying the gene *uidA*) or a mutant strain (with the insertionally inactivated version of the gene), the presence of  $\beta$ -glucuronidase in the digestive lumen dramatically increased the genotoxicity of IQ in the colon (118). Some dietary components have been associated with a decrease in  $\beta$ -glucuronidase activity, including decreased dietary oligosaccharide diet content in turkeys (127) and hesperetin supplementation in rats. Hesperetin is a citrus flavonoid, abundant in orange and grape juices (12). However, a crossover feeding trial that studied the effect of fruit and vegetable intake in 63 healthy women and men ages 20 to 40 yr (167) and a study that evaluated the impact of a regular consumption of yogurt on the composition and metabolism of the human intestinal microbiota did not show a significant effect of either fruit/vegetables or yogurt on  $\beta$ -glucuronidase activity of the intestinal microbiota (8).

**Production of aromatic amines by azoreductases.** Bacterial azoreductases metabolize a number of compounds including azo colorants and drugs (for example, Sulfazalazine, a drug used for the treatment of inflammatory bowel diseases) to produce aromatic amines, which may have carcinogenic activities (106). Water-soluble azo dyes are metabolized by the intestinal microbiota, whereas water-insoluble azo dyes are metabolized by reductases in the liver (58). Azoreductases catalyze the reductive cleavage of azo groups (-N=N-). Different types of bacterial azoreductases have been isolated and characterized. Their size range varies from 28 to 62 kDa; they can use NADH or NADPH as electron donor, may or may not require flavin mononucleotide as cofactor, and can be aerobic or oxygen sensitive (55, 124). Intestinal bacteria reported to have azoreductase activity include *Acidaminococcus fermentans*, *Enterobacter aerogenes*, *Bacillus* sp., *Bacteroides* sp., *Bacteroides fragilis*, *B. thetaiotaomicron*, *Bifidobacterium adolescentis*, *B. infantis*, *Butyrivibrio* sp., *Citrobacter* sp., *Clostridium nexile*, *C. clostridioforme*, *C. paraputrificum*, *C. ramosum*, *C. sporogenes*, *Coprococcus catus*, *Enterococcus faecalis*, *E. coli*, *Eubacterium* sp., *Eubacterium aerofaciens*, *E. bifforme*, *E. hadrum*, *Fusobacterium* sp., *Faecalibacterium prausnitzii*, *Klebsiella aerogenes*, *Lactobacillus* sp., *Lactobacillus catenaforme*, *Peptococcus prevotii*, *Peptostreptococcus productus*, *Pneumococcus* sp., *Proteus* sp., *Proteus vulgaris*, *Pseudomonas* sp., *Pseudomonas aeruginosa*, *P. pyrocyanea*, *Ruminococcus bromii*, *Salmonella paratyphi*, *S. typhimurium*, *Shigella dysenteriae* (Type 1), *Staphylococcus aureus*, *Streptococcus faecalis*, *Enterococcus faecalis*, *E. faecium*, *S. haemolyticus*, and *Veillonella parvula* (reviewed by Chung and Stevens, Ref. 58). A more recent study confirmed azoreductase activity in 17 out of 40 microbial intestinal species using microarrays. Among these 17 species, *Clostridium perfringens*, *Clostridium clostridioforme*, *Enterococcus faecalis*, *Ruminococcus obeum*, and *Bifidobacterium adolescentis* showed the highest azo dye reduction activity (275). Although azoreductase activity has been linked to cancer and to members of the intestinal microbiota, one study showed that there was no correlation between azoreducer strains isolated from healthy adults vs. strains of the same species isolated from patients with colon cancer (186). This study highlights the need for further characterization of intestinal bacteria and their metabolic activities.

**Production of aromatic amines by nitroreductases.** Another source of aromatic amines results from the metabolism of aromatic nitro compounds like dinitro toluene, nitrobenzenes, and nitropyrenes by nitroreductases. Two types of bacterial nitroreductases have been described on the basis of reduction of the nitro groups of polynitroaromatic compounds through the one- or the two-electron mechanism. Type I nitroreductases are oxygen-insensitive and catalyze the sequential reduction of nitro groups through the addition of electron pairs from NAD(P)H to produce the nitroso, hydroxylamino, and amino derivatives. Type II nitroreductases are oxygen sensitive and catalyze the single-electron reduction of the nitro group to produce a nitro anion radical, which can be reoxidized aerobically to the original structure with the concomitant production of the superoxide anion in a futile cycle (reviewed by Roldan et al., Ref. 225). The type I nitroreductase NfsB has been described in a strain of *E. coli* isolated from the jejunum of rats. NfsB catalyzes the nitroreduction of nitrobenzodiazepines, which are sedative-hypnotic drugs used for the treatment of anxiety (151). Homologs of NfsB can be found in species of *Neisseria*, *Desulfovibrio*, *Shigella*, *Salmonella*, *Enterobacter*, *Citrobacter*, *Klebsiella*, *Vibrio*, *Pseudomonas*, *Shewanella*, *Flavobacteria*, and *Shigella*. Also, a *P*-nitrobenzoate reductase, PnbA, has been recently characterized in *Lactobacillus plantarum* WCFS1. This enzyme showed low identity with nitroreductases from enterobacteria such as *E. cloacae*, but homologs were found in *Lactococcus lactis*, *L. gasseri*, *L. antri*, *L. sakei*, *L. fermentum*, and *L. vaginalis*, among others (107). The significance and role of different types of nitroreductases in the generation of carcinogenic compounds cannot be assessed at this time. As more intestinal bacterial genomes are sequenced, it should be possible to test whether there is a relationship between specific nitroreductases and the generation of toxic compounds.

**Generation of acetaldehyde.** The first step in the metabolism of ethanol is the oxidation to acetaldehyde by alcohol dehydrogenases (ADHs). Acetaldehyde is then metabolized to mainly acetate and NADH by acetaldehyde dehydrogenases (ALDHs). Acetaldehyde is considered a potent carcinogenic compound, and alcohol consumption has been linked to cancer of the oral cavity, pharynx, esophagus, liver, colon, rectum, and breast in women (29, 238). Human ADHs are encoded by at least seven genes and comprise five different classes (116). The different isozymes are differentially active in different tissues. A study in rodents detected expression of ADH1, ADH3, and ADH4 as well as ALDH2 in the mucosal layer of the GI tract, with characteristic regional differences (279). Given the contribution of human ADHs to acetaldehyde production, it is not entirely possible to correlate microbial producers of acetaldehyde in the GI tract with CRC. However, a study by Seitz et al. (239) showed that mucosal acetaldehyde levels in rodents were significantly lower in germ-free compared with conventional animals. In fact, a search for enzymes with the EC number 1.1.1.1 (ADH) in the Integrated Microbial Genomes-Human Microbiome Project (IMG/HMP) system (163) indicated the presence of this domain in the majority of annotated microbial genomes of GI origin in the database (125/185) with several organisms encoding more than one copy of the gene. The genera of GI origin encoding putative ADHs are *Akkermansia*, *Anaerofustis*, *Anaerostipes*, *Anaerotruncus*, *Bacillus*, *Bacteroides*, *Bifidobacterium*, *Blautia*, *Bry-*

*antella, Burkholderia, Citrobacter, Clostridiales, Clostridium, Collinsella, Coprococcus, Desulfovibrio, Dorea, Enterobacter, Enterococcus, Escherichia, Eubacterium, Faecalibacterium, Finegoldia, Helicobacter, Klebsiella, Lactobacillus, Laribacter, Leuconostoc, Mitsuokella, Parabacteroides, Prevotella, Proteus, Providencia, Ruminococcus, Streptococcus, Subdoligranulum, Vibrio, Victivallis, and Yersinia.*

**Desulfation of BAs.** Sulfation of BAs is an important metabolic pathway to detoxify and promote fecal secretion of BAs because BA-sulfates are more water soluble and undergo limited enterohepatic recirculation (224). Sulfation is catalyzed by a group of enzymes called sulfotransferases being monosulfation at the 3-OH position predominant in humans (7, 101). The role of the commensal microbiota in BA desulfation was suggested by Robben et al. (223) in a study that showed in rats that microbial desulfation of intraperitoneally injected ( $^{24-14}\text{C}$ )tauroolithocholate-3-sulfate caused a fivefold decrease in the fecal plus urinary excretion rate of the isotope to approximately that found for sulfated ( $^{24-14}\text{C}$ )tauroolithocholate. A study conducted to investigate hydrolytic and reductive enzymes involved in formation of genotoxic metabolites in the large intestine identified *C. perfingens* and species of bacteroides and bifidobacteria as members of the commensal microbiota with arylsulfatase activity (172). The presence of arylsulfatases in the following genera was confirmed by searching the IMG/HMP system: *Anaerotruncus, Bacteroides, Bifidobacterium, Blautia, Bryantella, Citrobacter, Clostridium, Collinsella, Escherichia, Holdemania, Klebsiella, Parabacteroides, Proteus, Providencia, Roseburia, and Subdoligranulum.*

**Generation of ROS.** The results of recent studies suggest that the NADPH oxidase (NOX) family, notably NOXs and dual oxidases (DUOXs), are primarily involved in the generation of ROS by various nonphagocytic cells, including those of the gut epithelia (224). Oxidative stress damages cell components including proteins, lipids, membranes, and DNA. Types of DNA damage include depurination and depyrimidination, single- and double-stranded DNA breaks, base and sugar modifications, and DNA-protein crosslinks. The permanent modification of DNA resulting from the oxidative damage is one of the most important factors involved in mutagenesis that leads to carcinogenesis (3). In addition, large quantities of  $\text{H}_2\text{O}_2$  are produced and excreted by human tumor cells (255), a mechanism that may enhance tumor invasion and proliferation. On the other hand, because ROS have a role in promoting cell apoptosis, a number of anticancer agents act by generating ROS-induced tumor cell death (38, 143, 286). Studies have shown that commensal bacteria induce generation of ROS in gut epithelial cells and that these metabolites act as key messengers that modulate the protein degradation machinery of several essential signaling components, which in turn influence diverse physiological processes of the host cells including cell proliferation and inflammation (141, 224). Therefore, ROS-generating enzyme systems have been studied as potential approaches to both prevent and treat cancer (188).

#### *Efficacy of Probiotics of the LAB in CRC and CRC Prevention*

The complex interaction between diet, normal intestinal microbiota, and health has promoted the development of strat-

egies that allow for the selective growth of beneficial microorganisms or probiotics. These bacteria include those of the *Bifidobacterium* and *Lactobacillus* genera, which are used as markers of stability of the normal human intestinal microbiota. Probiotics have drawn attention both as a way of managing disorders of the GI tract and as a result of their role in the modulation of the immune system (98, 152, 259). Probiotics are “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host” (219). The potential role of probiotics has been extensively reviewed (99, 198), and their beneficial effects include reinforcement of the natural defense mechanisms and protection against GI disorders. Probiotics have been successfully used to manage infant diarrhea (56, 93), food allergies (205), and IBD (32). A number of publications have reviewed the role of probiotics in chemoprevention of CRC (88, 153, 165). There is not a general consensus on the role of probiotics in CRC protection. In many cases this lack of agreement is due to the fact that researchers refer to probiotics as a group without considering 1) whether the probiotic tested is composed of one or more strains, 2) the species included in the probiotic preparation, 3) the preparation of the probiotic blend, e.g., the physiological state of the bacterial cultures at the moment of lyophilization or drying, 4) the age of the probiotic preparation (was the preparation kept cool, dry, and protected from the light?), and 5) whether the doses administered (CFU/ml) were adequate and comparable. There is, however, a general agreement that specific probiotic strains can beneficially affect metabolic activities that occur in the GI tract and that they enhance the host’s immune response. Evidence for the effect of probiotics in animal and human studies as well as in vitro studies is presented below.

**Animal studies.** Table 1 reviews the effect of probiotic intervention in animal models of CRC. Chemically induced (autochthonous) tumors in rodents are considered good test models to obtain results transferable to the clinical situation (10) with 1,2-dimethylhydrazine (DMH) being the most common carcinogen used in colon cancer induction. DMH is extensively metabolized in vivo, and toxicity has been ascribed to metabolism-generated reactive intermediates, such as alkyl-diazonium ions, carbon-centered radicals, and ROS (92). From the studies detailed in Table 1, we can generally conclude that the beneficial effects of probiotics are species and strain dependent and that the LAB have to be alive. In addition, research suggests that probiotics are more effective in high-fat diets, an interesting fact considering that CRC has been linked to high consumption of red and processed meats and indirectly to high consumption of fat. A clear reduction in aberrant crypt foci (ACF) was mostly observed with prebiotic and synbiotic preparations (nutritional supplements combining probiotics and prebiotics); however, the effect of probiotics alone is not as clear. Finally, beneficial effects were observed when probiotics were fed before and early during carcinogen treatment but not later in the studies.

**Studies in humans.** A number of publications report beneficial (75, 197) or detrimental (100) effects of consuming probiotics to treat or prevent diarrhea induced by cancer therapies. However, the number of clinical trials involving the use of probiotics for prevention or treatment of CRC is scarce (Table 2). With the overall goal of contributing to the formulation of improved dietary advice, the EU-sponsored project SYNCAN aimed to evaluate the potential CRC-preventing activity of a

Table 1. Effect of probiotic intervention in animal models of colorectal cancer

Model	Treatment	Results	Reference
Sprague-Dawley rats, AOM-induced colon cancer model	<i>Bifidobacterium lactis</i> (DSM Food Specialties, Australia) plus "resistant starch" (RS)	RS plus <i>B. lactis</i> significantly protect against the development of colorectal cancer in the rat-AOM model.	145
Male Wistar SPF rats, DMH-induced colon cancer model	Moderate or intense physical exercise, alone or in combination with the consumption of a soy product fermented with <i>Enterococcus faecium</i>	Soy product and the practice of physical exercise (intense or moderate) were incapable, separately or combined, of inhibiting the formation of ACF in DMH-induced rats.	246
Rat AOM-induced colon cancer model	<i>Lactobacillus rhamnosus</i> GG and <i>Bifidobacterium lactis</i> Bb12, alone or plus inulin enriched with oligofructose (SYN)	SYN supplementation in carcinogen-treated rats primarily modulated immune functions in the gut-associated lymphoid tissue, coinciding with a reduced number of colon tumors.	226
Sprague-Dawley rats, AOM-induced colon cancer model	Not specified "novel synbiotic preparation".	Significantly reduced ratio of ACF/colon and of ACF per colon and per each single focus	64
Rat AOM-induced colon cancer model	<i>Lactobacillus casei</i> strain Shirota (LcS)	The number of rats with colon cancers and the number of colon cancers per rat were significantly decreased in the rats that had consumed the LcS diet. LcS inhibited chemically induced colon carcinogenesis in the rat.	284
Male Fisher 344 weanling rats, AOM-induced colon cancer model	<i>Bifidobacterium longum</i> BB536 (Morinaga Milk Industry, Japan) alone or plus lactulose (SYN).	There was a significant reduction in the total number of ACF in the colons of the rats consuming <i>B. longum</i> , lactulose, or SYN diets compared with the control. There was no significant difference between the <i>B. longum</i> and lactulose groups in total number of ACF.	52
Male Fisher 344 weanling rats, AOM-induced colon cancer model	<i>Bifidobacterium longum</i>	Lyophilized cultures of <i>B. longum</i> significantly inhibited the ACF formation and the crypt multiplicity in the colon. A significant decrease in the fecal bacterial $\beta$ -glucuronidase was also observed.	137
Male Wistar SPF rats, DMH-induced colon cancer model	<i>Lactococcus lactis</i> NZ9000	No differences in tumor incidence, multiplicity, dimensions, and stage in the colonic mucosa were observed among the groups.	148
Male Fisher 344 rats, AOM-induced colon cancer model	<i>Lactobacillus</i> GG, <i>L. delbrueckii</i> subsp. "rhamnosus", and <i>Bifidobacterium lactis</i> Bb12 (Chr. Hansen, Horsholm, Denmark), alone or plus raitilose (SYN)	After 16 wk of feeding, SYN significantly increased ACF multiplicity. After 32 wk, SYN significantly decreased intestinal tumors. Crypts with scarce or absent mucins were identified (MDF), visible in all AOM-treated rats, and correlated with tumor induction. There were fewer MDF/colon than ACF, and they were histologically more dysplastic than mucinous lesions identified as ACF in high-iron diamine Alcian blue-stained colon.	44
Male Fisher 344 rats, AOM-induced colon cancer model	<i>Bifidobacterium lactis</i> (Bb12) and <i>Lactobacillus rhamnosus</i> (LGG), alone or plus raitilose	31 wk after AOM, rats treated with raitilose had a significantly lower number of tumors (adenomas and cancers). Nonsignificant effect of probiotics in reducing tumors was also observed. Cecal short-chain fatty acids (SCFA) were higher in the groups treated with raitilose. Apoptosis was increased in the normal mucosa of the probiotic group, whereas no variation was observed in the tumors. Colonic proliferation was lower in the raitilose group.	86
Sprague-Dawley rats, AOM-induced colon cancer model	<i>Bifidobacterium longum</i> , <i>Lactobacillus acidophilus</i> , and <i>Lactobacillus casei</i> selected to inhibit MNNG and DMH-induced genetic damage to the colon	A significant decrease in AOM-induced colonic ACF was observed in rats fed a high-fat diet (corn oil) given <i>L. acidophilus</i> or inulin. In a concurrent group of animals fed a low-fat diet, no significant decrease in ACF was observed.	30
Sprague-Dawley rats, DMH-induced colon cancer model Diet: high-fat semipurified (AIN-93)	<i>Lactobacillus acidophilus</i> Delvo Pro LA-1, <i>Lactobacillus rhamnosus</i> GG, <i>Bifidobacterium animalis</i> C5CC1941, and <i>Streptococcus thermophilus</i> DD145	Large intestinal tumor burden and mass were significantly lower for rats treated with <i>L. acidophilus</i> , <i>L. acidophilus</i> and <i>L. rhamnosus</i> GG but not <i>B. animalis</i> and <i>S. thermophilus</i> were reisolated from feces, indicating their survival through the GI tract.	174

Continued

Table 1.—Continued

Model	Treatment	Results	Reference
Male Fisher 344 rats, AOM-induced colon cancer model	<i>Lactobacillus acidophilus</i> NCFM	NCFM significantly suppressed AOM-induction of colonic ACF, in terms of total number, as well as crypt multiplicity and number of ACF/cm <sup>2</sup> colon. NCFM inhibited AOM-induced colonic ACF formation in a dose-dependent manner. A significant dose-dependent reduction of cecal $\beta$ -glucuronidase activities was observed.	216
Male Fisher 344 rats, AOM-induced colon cancer model	<i>Bifidobacterium longum</i> 25 (Unilever Research Laboratory, Vlaardingen) alone or plus inulin (SYN)	<i>B. longum</i> or inulin were associated with a decrease in AOM-induced small ACF (1-3 aberrant crypts per focus). SYN administration resulted in more potent inhibition of ACF (80% inhibition of small ACF) and decreased the incidence of large ACF (>4 aberrant crypts per focus).	230
Rats, DMH-induced colon cancer model	<i>Lactobacillus acidophilus</i> , <i>Lactobacillus casei</i> (NDRI, Karnal), and curd culture <i>Lactococcus lactis</i> biovar. Diacetyllactis DRC-1	A significant reduction in DNA damage assessed by the comet assay was observed in the probiotic curd group.	139
Male Fisher 344 rats, AOM-induced colon cancer model	Nonspecified EPS-producing and non-EPS-producing cultures	Rats fed diets supplemented with fermented milk made with 2 EPS-positive and 1 EPS-negative strains had significantly lowered incidence of colon tumor and colon tumor multiplicity. Cyclooxygenase-2 enzyme activity was significantly lower in the colon tissue of rats fed diets containing milk fermented with 4 EPS-producing and 1 nonproducing cultures than that in rats fed diets supplemented with acidified milk. No relationship was found between rheological properties or level of ropiness of fermented milk and its chemopreventive effect.	210
Rats, MNNG- and DMH-induced colon cancer model	<i>Lactobacillus acidophilus</i> (from commercially available yogurt), <i>Lactobacillus gasseri</i> P79, <i>Lactobacillus confusus</i> DSM20196, <i>Streptococcus thermophilus</i> NCIM 50083, <i>Bifidobacterium breve</i> and <i>Bifidobacterium longum</i> (from human infant stool)	Pretreatment orally with lactic acid bacteria (LAB) on 4 consecutive mornings before DMH gavage (8 h after the last LAB application) revealed that <i>L. acidophilus</i> , <i>L. confusus</i> , <i>L. gasseri</i> , <i>B. longum</i> , and <i>B. breve</i> inhibited the genotoxic effect of DMH. One of four <i>S. thermophilus</i> and one of three <i>Lactobacillus delbrueckii</i> ssp. <i>bulgaricus</i> strains were also protective. Heat-treated <i>L. acidophilus</i> did not inhibit DMH-induced genotoxicity.	206
Male Fischer 344, DMH-induced colon cancer model 20% or 5% corn oil diet	<i>Lactobacillus rhamnosus</i> GG	Significant decrease in the incidence of colon tumors and the number of small intestinal and colon tumors per tumor-bearing animal for rats fed a 20% corn oil diet. Animals fed a 5% corn oil diet had a lower tumor incidence and number of tumors resulting from the decrease in dietary fat. Decrease in tumor incidence or number of tumors was not seen when animals were fed the <i>Lactobacillus</i> after the ninth week of carcinogen treatment.	104
Male Sprague-Dawley rats, AOM-induced colon cancer model	<i>Lactobacillus acidophilus</i> , <i>Bifidobacterium adolescentis</i> , <i>Bacteroides fragilis</i> , <i>Escherichia coli</i> and <i>Clostridium perfringens</i>	The number of ACF 5 wk after the start of the experiment was decreased in the rats treated with <i>L. acidophilus</i> and <i>C. perfringens</i> . The inhibitory effect of <i>L. acidophilus</i> is due to the enhanced removal of O6-methylguanine from the colon mucosal DNA.	15
Rats, DMH-induced colon cancer model	<i>Bifidobacterium</i> sp Bio (Danone strain 173010)	Significant reduction of aberrant crypts	1

Continued

Table 1.—Continued

Model	Treatment	Results	Reference
Male Wistar SPF rats, DMH-induced colon cancer model	<i>Bifidobacterium</i> sp. (Chr. Hansen, Inc., Milwaukee, WI), <i>L. acidophilus</i> , alone, in combination and plus fructooligosaccharides (FOS)	Bifidobacteria + FOS administration significantly decreased the number of aberrant crypts that developed. Bifido-FOS treatment led to significantly fewer aberrant crypts and ACF than the Bifido-Basal treatment. The Skim-FOS group had significantly more cecal bifidobacteria than the Skim-Basal group and significantly fewer <i>C. perfringens</i> than the Skim-Basal and Bifido-Basal. The number of aberrant crypts was not significantly different among the groups when <i>L. acidophilus</i> was added. However, the number of <i>C. perfringens</i> was significantly decreased by the addition of bifidobacteria, <i>L. acidophilus</i> , or the combination of the two.	91
F344 rats fed the high-fat diet containing 2-amino-3-methylimidazo(4,5-f)quinoline (IQ)	Lyophilized cultures of <i>Bifidobacterium longum</i>	<i>B. longum</i> significantly inhibited the IQ-induced incidence (percentage of animals with tumors) of colon (100% inhibition) and liver (80% inhibition) tumors and multiplicity (tumors/animal) of colon, liver, and small intestinal tumors in male rats. Dietary supplementation of <i>B. longum</i> resulted in a significant inhibition of colon, small intestine, and liver tumor incidences in male rats ( $P < 0.05$ ). In female rats, dietary supplementation of <i>B. longum</i> also decreased mammary carcinogenesis to 50% and liver carcinogenesis to 27% of those observed in animals fed the control diet, but the differences did not reach a statistical significance.	218
Male Fischer 344, DMH-induced colon cancer model	Viable <i>Lactobacillus acidophilus</i>	The colon cancer incidence after a 20-wk induction period was lower in the animals receiving <i>L. acidophilus</i> (40% vs. 77% in controls), but no difference in incidence was discerned after a 36-wk period.	103
Fischer 344, DMH-induced colon cancer model	Milk fermented with <i>Streptococcus thermophilus</i> or <i>Lactobacillus bulgaricus</i>	Feeding fermented milks altered the metabolism of 1,2-dimethylhydrazine and shifted the target organ from the ear duct to the small intestine. In addition, the colon tumor distribution for the fermented-milk groups appeared to shift toward the anus.	244
Male Fisher 344 rats, AOM-induced colon cancer model modified AIN-76A diet	Lyophilized cultures of <i>B. longum</i>	Significant suppression of colon tumor incidence and tumor multiplicity and also reduced tumor volume were seen. Results also revealed that ingestion of <i>B. longum</i> significantly inhibited AOM-induced cell proliferation, ODC activity, and expression of ras-p21 oncoprotein.	248
ICR mice inoculated with sarcoma-180 intraperitoneally and BDF1 mice inoculated with L1210 leukemia intraperitoneally	<i>Lactobacillus casei</i> YIT 9018 (LC 9018)	Intravenous injection of LC 9018 markedly inhibited the growth of subcutaneously inoculated sarcoma-180. This organism was also effective against methylcholanthrene-induced syngeneic MCA K-1 tumor in BALB/c mice. The antitumor activity of LC 9018 was reduced by treatment with carrageenan, an antimacrophage agent and was also observed in T-cell-deficient athymic nude mice.	130
Germfree (GF) mice human-flora-associated (HFA) mouse treated with AAC	<i>Streptococcus faecalis</i> T-110, <i>Clostridium butyricum</i> TO-A, and <i>Bacillus mesentericus</i> TO-A mixture (Biothree, TOA Pharmaceutical Tokyo, Japan)	The mean level of the DNA adducts in the colonic epithelium of the probiotic group was significantly lower than that of control group.	117
DMH-treated BALB/c mice	<i>Lactococcus lactis</i> expressing catalase	Animals that received the catalase-producing <i>L. lactis</i> had a significantly lesser extent of colonic damage and inflammation.	72
DMH-treated Male albino rats of Wistar strain	<i>Lactobacillus acidophilus</i> , <i>Lactobacillus casei</i> ssp. <i>casei</i> , and dahi culture DRC-1 ( <i>Lactococcus lactis</i> ssp. biovar. diacetylactis NCDC-60)	Addition of Ac Dahi to wheat bran acted synergistically against intestinal carcinogenesis.	140

AOM, azoxymethane; ACF, aberrant crypt foci; DMH, 1,2-dimethylhydrazine; MDF, mucin-depleted foci; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; IQ, 2-amino-3-methylimidazo[4,5-f]quinoline; AAC, 2-amino-9H-pyrido[2,3-b]indole (2-amino- $\alpha$ -carboline); EPS, exopolysaccharide; ICR, imprinting control region.

Table 2. Clinical trials of probiotic intervention for prevention or treatment of colorectal cancer

Model	Treatment	Results	Reference
20 human volunteers	R/DB/PC/C. Resistant starch (RS) containing high-amylose maize starch and <i>Bifidobacterium lactis</i> (alone or in combination).	The synbiotic intervention fostered a unique fecal stream bacterial community, with greater proportion of patients harboring fecal <i>Lachnospiraceae</i> spp. No significant effect on epithelial proliferation or crypt height was observed, and fecal SCFA concentrations did not change between interventions or when compared with baseline (probably due to low doses of RS).	282
38 healthy men	R/DB/PC/C. <i>Lactobacillus rhamnosus</i> LC705 (LC705) together with <i>Propionibacterium freudenreichii</i> ssp <i>shermanii</i> JS (PJS)	Administration of LC705 and PJS was followed by an increase in the fecal counts of lactobacilli and propionibacteria and a decrease in the activity of $\beta$ -glucosidase.	111
150 patients diagnosed with colorectal cancer	<i>Lactobacillus</i> GG	<i>Lactobacillus</i> GG supplementation was well tolerated and may reduce the frequency of severe diarrhea and abdominal discomfort related to 5-FU-based chemotherapy.	197
398 men and women presently free from tumor who had had at least 2 colorectal tumors removed	Wheat bran and/or <i>L. casei</i>	<i>L. casei</i> prevented atypia of colorectal tumors.	123
31 subjects undergoing elective colorectal resection for cancer	Mixture of <i>Bifidobacterium longum</i> (BB536) and <i>Lactobacillus johnsonii</i> (La1)	La1, but not BB536, adheres to the colonic mucosa and affects intestinal microbiota by reducing the concentration of pathogens and modulates local immunity.	96
37 patients with colon cancer and 43 polypectomized patients	R/DB/PC. A synbiotic preparation- oligofructose-enriched inulin (SYN1) + <i>Lactobacillus rhamnosus</i> GG (LGG) and <i>Bifidobacterium lactis</i> Bb12 (BB12)	Numbers of <i>Bifidobacterium</i> and <i>Lactobacillus</i> increased, and <i>Clostridium perfringens</i> decreased. There was a reduction of colorectal proliferation, and the capacity of fecal water to induce necrosis in colonic cells and improve epithelial barrier function in polypectomized patients. Synbiotic consumption prevented an increased secretion of interleukin-2 by peripheral blood mononuclear cells in polypectomized patients and increased the production of interferon- $\gamma$ in cancer patients.	213
100 patients with colorectal carcinoma scheduled for radical colectomy randomly divided into control ( $n = 50$ ) and probiotics groups ( $n = 50$ )	Probiotics containing <i>Lactobacillus plantarum</i> (CGMCC No. 1258, $10^{11}$ CFU/g), <i>Lactobacillus acidophilus</i> (LA-11, $10^{10}$ CFU/g), and <i>Bifidobacterium longum</i> (BL-88, $10^{10}$ CFU/g)	Intervention lasted 16 days (6 days preoperatively and 10 days postoperatively). The total probiotic daily dose was $2.6 \times 10^{14}$ CFU/g. The treatment improved the integrity of the gut mucosal barrier and balance of the gut microbiota and decreased rate of post surgical infection.	154

R/DB/PC/C: Randomized, double-blind, placebo-controlled, crossover trial.

synbiotic product in human volunteers. Preliminary in vitro studies first identified synbiotic combinations offering the greatest competitive advantages in the colon ecosystem and established the anticarcinogenic effect of the most promising synbiotic combination in a rat colon cancer model (207, 272). From these studies, Pool-Zobel et al. (207) concluded that the fermentation products of inulin-type fructans by the gut microbiota, specifically butyrate and propionate, are capable of inhibiting histone deacetylases and growth of colon tumor cells. Despite this finding, the mechanisms involved in a potential effect of probiotics in colon cancer are not yet fully characterized.

#### Designing Tailored Probiotics

From the many studies published in the field we could conclude that, when it comes to probiotics there is no “one size fits all”. The vast majority of studies involving probiotics suggests a role for probiotics mainly to prevent CRC. However, there is gray area between prevention and early stages of CRC that could benefit from treatment with selected probiotic

strains. From animal studies it is evident that species of *Bifidobacterium* and *Lactobacillus*, specifically *B. lactis*, *B. longum*, *L. acidophilus*, *L. casei*, and *L. rhamnosus* have a beneficial impact on preventing and reducing the number of ACFs in mice (see Table 1 for references), whereas *Enterococcus* and *Lactococcus* are mostly ineffective. However, specific activities such as butyrate production are present in *E. durans* (217), and in one study DMH-treated mice that received catalase-producing *L. lactis* had a lower extent of colonic damage and inflammation (72). This one example indicates that there might be two paths to achieve a full probiotic effect; one is to genetically modify one or more than one strains to combine and include multiple beneficial activities. This strategy is definitely showing some promise, as a number of studies using genetically modified probiotic strains have demonstrated beneficial effects. A Phase I clinical trial was conducted with a *Lactococcus lactis* strain in which the thymidylate synthase gene was replaced with a synthetic sequence encoding mature human interleukin-10. Ten patients with Crohn’s disease were included and showed a decrease in

disease activity (34). The fact that the recombinant bacterium requires thymidine to grow prevents its widespread distribution in nature and provides an effective containment strategy. Other studies have shown that expression of the MnSOD in *L. plantarum*, *L. lactis*, and *L. gasseri* improved probiotic strain survival (39, 41) and reduced colitis symptoms (48). *B. breve* and *Lactococcus lactis* expressing the *bilE* gene from *Listeria monocytogenes* showed increased resistance to bile and were also recovered at significantly higher levels than control strains from the feces and intestines of mice (278). Alternatively, a carefully selected blend of strains may be the more appropriate approach given the controversy that might arise from the use of genetically modified organisms (42, 271). From the topics covered in this review we have identified microbial features with an effect, beneficial or otherwise, in CRC as well as essential characteristics needed by probiotic strains to survive and thrive in the GI tract.

**Survival and colonization of the specific intestinal microenvironment.** The GI tract has the dual tasks of absorbing nutrients and protecting the body from potentially harmful microorganisms. It possesses a unique structure with a massive surface area that enhances absorption and also houses the largest numbers of immune cells in the body (168). These characteristics create an exceptional environment that is able to host and shape the vast human intestinal microbiota. It is not surprising that the disruption of the intestinal environment by disease, diet, physical trauma, antibiotics, and other agents has a profound effect on the residing microorganisms and can create a microbial dysbiosis, which negatively affects the host's overall health.

**ACID RESISTANCE.** Chan et al. (53) showed that tumorigenic colon microenvironments had increased concentrations of lactate, phosphate, L-glycine, L-proline, L-phenylalanine, palmitic acid, marganic acid, oleic acid, stearic acid, uridine, 11,14-eicosadienoic acid, 11-eicosenoic acid, 1-O-heptadecylglycerol, 1-monooleoylglycerol, propyl octadecanoate, and cholesterol. The high lactate production rate of malignant cells was documented as early as 1956 (277) when Warburg proposed an energy deficiency in normal cells after suppression of their respiration, which "forces the cells to replace the irretrievably lost respiration energy in some way," thus increasing fermentation. The production of increased concentrations of lactate persists even in the presence of oxygen, a phenomenon occasionally called "aerobic glycolysis" or the "Warburg effect". On the contrary, production of lactate is repressed, and glycolysis is slowed down by environmental oxygen in most normal mammalian cells, which is referred to as the "Pasteur effect" (274). Consequently, resistance to lactate is an important feature when selecting for probiotic strains to increase their chances for survival in an inflamed/tumorigenic GI environment. The mechanisms involved in acid resistance by lactic acid bacteria include 1) proton pumps, of which the most prevalent are  $F_1F_0$ ATPases; 2) amino acid decarboxylation reactions, which result in the formation of biogenic amines; and 3) the urease and arginine deiminase pathways, which produce  $\text{NH}_3$  that combines with protons in the cytoplasm to produce  $\text{NH}_4^+$ , raising the internal pH (20, 64, 240). Additionally, we have recently shown that superoxide dismutases, involved in resistance to oxidative stress, can also protect some organisms against acid stress (40). Other systems involved in the resistance to acidic environments include 1) chaperones,

proteases, and heat shock proteins, which protect or degrade proteins if damaged; 2) DNA repair systems that excise errors or restart stalled replication forks; 3) transcriptional regulators such as two-component regulatory systems and  $\sigma$ -factors that induce minor or global responses; and 4) quorum-sensing-driven events. In addition, cells respond to acidic environments by altering architecture, composition, stability, and activity of their envelope (21, 36, 64, 240).

**RESISTANCE TO OXIDATIVE STRESS.** A number of studies have shown a significant role of oxidative stress in the initiation and growth of colorectal adenomas and demonstrated adaptation of CRC cells to oxidative stress (189, 194, 249). Consequently, enzymes that can detoxify ROS are desirable in probiotic strains. Oxidative stress can cause several types of damage to bacterial cells and has bacteriostatic and bactericidal effects (90, 287). Although oxygen by itself is unable to cause any damage to the cell, ROS are generated during cellular processes where  $\text{O}_2$  is partially reduced to water. These intermediates have a high oxidizing potential and thus are responsible for cellular oxygen toxicity (90). To offset the harmful effects of ROS, some LAB have evolved protective mechanisms that utilize antioxidant enzymes, such as superoxide dismutases and hydroperoxidases (i.e., catalases and peroxidases; or KatE and KatG), intracellular enzymes that scavenge superoxide radicals and hydrogen peroxide, respectively, preventing the formation of HO via Fenton chemistry (87). In *Streptococcus* and *Lactococcus* the elimination of ROS conform to a general bacterial antioxidative defense system because both genera possess MnSOD (209, 235). With a few exceptions (70, 138), most lactobacilli lack this general defense system. Some lactobacilli species developed an alternative nonenzymatic defense system that involves the accumulation of high intracellular concentrations of Mn(II), which can scavenge  $\text{O}_2^-$  (13, 14). Additionally, to minimize the potentially toxic effects of the oxygen, *Lactobacillus* contains enzymes such as NADH oxidases, NADH peroxidases, or pyruvate oxidases (102, 257) and the competitive advantage of very low iron growth requirement (82, 121).

**RESISTANCE TO BILE.** The role of bile acids as carcinogens in the GI tract has been recently recognized (see above). However, not all BAs are considered cytotoxic. Secondary BAs are the products of bacterial dehydroxylation, dehydrogenation, and sulfation, or  $7\alpha$ -dehydroxylation of deconjugated bile salts and have been shown to stimulate colorectal epithelial proliferation in animals (9) and, in high concentrations in feces, blood, and bile, have been linked to cholesterol gallstone disease and colon cancer (173). A higher conversion of primary to secondary BAs in the GI tract is strongly stimulated by a diet high in red meats and fat (24, 25). A general mechanism by which secondary bile acids may act to promote tumorigenesis is by generating ROS and reactive nitrogen species (RNS), which can lead to increased DNA damage and then an increase in the mutation rates (80). As discussed above,  $7\alpha$ -dehydroxylation activity is present in several species of the genus *Clostridium* (*C. absonum*, *C. scindens*, *C. bifementans*, *C. limosum*, and *C. hylemonae*) (221), whereas BSHs are widely distributed in the genera *Lactobacillus* and *Bifidobacterium*, members of the LAB, most of which are considered probiotics (23, 204). The importance of a probiotic blend resistant to BAs and capable of decreasing gut microbiota-mediated generation of secondary BAs is evident.

**ADHESION.** The ability of probiotics to adhere to GI mucus is of considerable importance in their ability to exert a modulatory effect in situ. Moreover, naturally adhesive strains are of relevance for continuous production of probiotics using biofilm technology (discussed below). Mucus adherence has been extensively studied in several strains of probiotic LAB (22, 131, 256) and has revealed a broad repertoire of proteinaceous and nonproteinaceous surface components involved in adhesion to epithelial cells through passive or steric forces and electrostatic and hydrophobic interactions. These components include lipoteichoic acids and specific structures such as external appendages covered by lectins (reviewed in Ref. 241). The adhesion of probiotic bacteria to epithelial cells has been shown to prevent the establishment of pathogens; however, adhesion per se does not warrant persistence in the GI tract (150). Several studies that characterized LAB from different origins have shown that the ability to adhere to epithelial cells is strain dependent. The human cell lines Caco-2 and a mucus-secreting derivative (HT29-MTX cells) have been extensively and successfully used to identify adherent strains (47, 62, 63, 199, 232, 233).

**COMPETITIVE EXCLUSION.** As stated above, probiotic strains with adhesive properties can compete with and prevent establishment of pathogenic bacteria by competitive exclusion. Although the mechanisms by which probiotics inhibit pathogenic activity are not entirely known, it has been shown that it goes beyond a mere physical barrier. Resta-Lenert and Barret (220) showed that exposure of cell monolayers to live but not heat inactivated probiotic *S. thermophilus* and *L. acidophilus* strains significantly limited adhesion, invasion, and physiological dysfunction induced by exposure to an enteroinvasive strain of *E. coli*. As stated in the previous section, several proteinaceous and nonproteinaceous surface components have been involved in adhesion to epithelial cells (see references above). The probiotic strains increased transepithelial resistance, which was accompanied by maintained (actin, zonulin-1) or enhanced (actinin, occludin) phosphorylation of cytoskeletal and TJ proteins. A similar effect has been demonstrated for a probiotic strain of *L. plantarum*, which had a protective effect against damage to the integrity of Caco-2 monolayers and the structure and distribution of TJ proteins by enteroinvasive *E. coli* (211). In addition to the above described mechanism of competitive exclusion, studies have indicated that specific probiotic strains can promote mucus secretion. Given that the mucus layer is the first line of defense against pathogens, increased mucus thickness can be beneficial in cases where the mucus layer has been compromised as a result of inflammation or disease (193).

*Activities that reduce generation of mutagenic or genotoxic compounds.* A role for the commensal gut microbiota in the generation of mutagenic, carcinogenic, and/or genotoxic compounds in the GI tract has been established (see *The Normal Intestinal Microbiota and the Microbiota Associated with CRC*). In contrast, some commensal and probiotic bacteria have the potential to reduce such compounds and may diminish cancer risk (112, 149) via enzymatic activities that produce bioactive molecules like short-chain fatty acids, which are the endproducts of carbohydrate fermentation (specifically resistant starches and dietary fiber) by anaerobic bacteria. Fecal concentrations of different short-chain fatty acids include acetate > propionate > butyrate in a molar ratio of ~60:20:20,

respectively (262). Butyrate has been regarded as the most important nutrient for colonocytes and also has a major role in regulation of cell proliferation and differentiation (reviewed in Ref. 281). Butyrate is produced mainly in the proximal colon, where substrate availability is higher, and hence this region of the colon has a lower pH, which is significant because the lower pH enriches this region with the more acid-resistant LAB strains. A number of studies have shown that butyrate inhibits human colon carcinoma cell proliferation and induces apoptosis in human colon carcinoma cells (79, 195, 231). Additionally, butyrate possesses possible anticarcinogenic effects by suppressing the COX-2 expression in HT-29 and in Caco-2 cancer cell lines (190, 261) and promoting expression of differentiation markers such as alkaline phosphatase in colonic cell lines (78, 196). Butyrate also induces expression of the host's glutathione-S-transferases (GST) and other stress response genes (recently reviewed in Ref. 237). GSTs catalyze the reaction between glutathione and lipophilic compounds with electrophilic centers, which leads to the deactivation of toxic compounds, xenobiotics, and products of oxidative stress (81). Bacterial pathways of butyrate production have been characterized in *Clostridium acetobutylicum*, a solventogenic bacteria of technological importance, and more recently in *Butyrivibrio fibrisolvens*, a butyrate producer that has been found in the GI tract of humans, dogs, and cats (16, 17). The pathway for butyrate production involves two phases; the first converts acetyl-CoA to butyryl-CoA and requires four enzymes, thiolase,  $\beta$ -hydroxybutyryl-CoA dehydrogenase, crotonase, and butyryl-CoA dehydrogenase. Subsequently, butyryl-CoA is converted into butyrate via two alternative mechanisms, either a phosphotransbutyrylase and butyrate kinase convert butyryl-CoA to butyrate with the intermediate formation of butyryl-phosphate, or a butyryl-CoA:acetate CoA-transferase transfers the CoA moiety to external acetate, which leads to the formation of acetyl-CoA and butyrate (177, 185). Among LAB, no butyrate producers have been isolated in the genera *Lactobacillus*; however, genome sequencing of probiotic lactobacilli strains has shown the presence of the butyrate kinase gene in *L. casei* (46, 169). Additionally, a bacterial gut isolate identified as *Enterococcus durans* has been shown to be able to synthesize and secrete butyrate (217). Other bioactive compounds with cancer-preventive properties generated by microbial enzymes are equol (produced from the main isoflavin in soy, daidzein, see above) and conjugated linoleic acids (recently reviewed in Ref. 69).

BSHs deconjugate bile salts by the enzymatic hydrolysis of the C-24 *N*-acyl amide bond linking bile acids to their amino acid conjugates. They are in the choloylglycine hydrolase family (EC 3.5.1.24) and are widely distributed in gut microbial species (see above). The presence of this enzyme is correlated with resistance to bile in the GI tract and, hence, persistence and activity of probiotics in situ although some authors have questioned the beneficial effects of bacterial BSHs on the host (23, 201).

Probably the single most important bacterial enzymes correlated with cancer prevention are the ones with antioxidative activities because it has been extensively proven that exposure to ROS, RNS, or reactive lipid peroxidation products damages cellular DNA, causing oncogenic mutations or epigenetic changes (189). Hence, bacterial antioxidant enzymes are involved in both persistence of the probiotic in GI tract (see

RESISTANCE TO OXIDATIVE STRESS) and prevention of DNA damage by products of the oxidative metabolism. In this respect, studies have showed anti-inflammatory properties of *Lactobacillus gasseri*, *L. plantarum*, and *L. lactis* expressing manganese superoxide dismutase using the interleukin 10-deficient mouse or trinitrobenzene sulfonic acid rat models of colitis (48, 110).

Although specific aglycones have been reported to be carcinogenic in mice (see *Production of aglycones*), equol [7-hydroxy-3-(4'-hydroxyphenyl)-chroman] is an example of health-promoting aglycone. It is generated by the microbiota in response to soy isoflavone intake in some but not all humans and exhibits a wide range of biological properties (126, 242). In soybeans and nonfermented soy products, daidzein (one of the main isoflavonoids) occurs only in small amounts as aglycone (less than 5% of total daidzein), whereas the rest is mainly constituted of nonabsorbable, biologically inactive glycosides, acetylglycosides, or malonylglycosides (243). After ingestion, malonylglycosides are partially hydrolyzed in the small intestine, but a considerable portion of isoflavonoids reach the colon to be metabolized by specific bacterial groups. The bioactive compound equol, aglycone derived from daidzein (4',7-dihydroxyisoflavone) exclusively by bacterial enzymatic activities in the colon, has been found to exhibit antiandrogenic activities, inhibiting prostate cancer development (155). Conversely, a recent study reported that in mice early exposure to equol was not chemopreventive against mammary tumors induced by 7,12-dimethylbenz(a)anthracene although for R-(+)equol there was a trend toward delaying tumor formation (37). It is not clear which bacterial species are involved in equol production in the colon. One study identified a mixed microbial culture consisting of *Enterococcus faecium*, *Lactobacillus mucosae*, *Finexgoldia magna*, and *Veillonella* sp. (73) and, more recently, a new species of the genus *Eggerthella* (285) as equol producers. Another example of aglycones with potential anticancer activities is resveratrol-aglycone, which reduced the number of aberrant crypt foci/mouse in CF-1 mice (134). Table 3 lists the probiotic features of potential impor-

tance in CRC prevention and the bacterial activities previously linked to increased risk of CRC.

*Production of probiotics: improving the survival and efficacy of strains.* In addition to desirable metabolic characteristics, probiotic strains or blends of strains should also be selected for high stability, viability, and survival to ensure that a sufficient number of viable bacteria will reach the GI tract (108). Improving probiotic cell survival during the production process itself is also important to reduce the uncertainty and variability in probiotic viability and improve consistence in biological effects. It is also essential to define the dose of probiotics that guarantees at least a transient modification of the homeostatic equilibrium of the gut microbiota. Variables like strain viability, associated strains, survival in the GI tract, and interactions with other bacteria and with the host must be assessed during experimental development and validation of probiotic blends, while the dose to administer will be a function of the probiotic behavior after assessment of the mentioned variables. Traditionally, the production approach to generate starter cultures has been to control and maintain a specific physiological state of the strains. The physiological state concept was introduced by Malek (160) and has been debated for decades. Nowadays, it is accepted that terms as "young cells", "old cells", "midlog phase cells", and "stationary-phase cells" do not adequately define the age of bacterial cultures because the age of the cells can only be defined in relation to their inherent rates of cell division. The age of bacterial cultures during batch cultivation cannot be controlled, and consequently the environmental conditions are always transient. An improved approach to study and produce probiotics is to unequivocally control their physiological state by growing strains in continuous system under steady-state or pseudo-steady-state conditions. In this condition, rates of cell division are maintained constant, which enables the study of specific growth conditions in an environmental equilibrium. Therefore, continuous open culture systems must be selected for production of probiotics to eliminate variations in biological performance. Continuous open culture systems include biofilm operational systems that reproduce more closely the natural behavior of bacterial strains. Biofilm formation is a natural process in which microbial cells of single or multiple species adsorb to support without chemicals or polymers to entrap the bacteria. Microbial biofilms normally exhibit modified patterns of growth rate and gene expression, and their physiology and architecture have been extensively reviewed (67, 136, 144).

*GI in vitro systems to evaluate new probiotics.* The process of validating new probiotic alternatives to manage growing pathologies associated to Western diets includes preclinical assessments (biochemical and cellular assays) and animal toxicity testing before validation in human clinical trials. An increasing number of promising probiotic candidates fail in phase III clinical trials, which highlights the need for a better method to accurately evaluate the absorption, distribution (stability or viability in case of living bacteria), metabolism, elimination, and toxicity (ADMET) of new probiotic candidates early in the validation process (4). Two preliminary methods commonly used to determine the toxicological and pharmacological profiles of potential drugs are in vitro cell cultures and animal models. Single cell type monolayer cell cultures cannot measure the systemic changes caused by the

Table 3. Features in colorectal cancer prevention and increased risk

Characteristics
<i>Probiotic features of potential importance in colorectal cancer prevention</i>
1. Bile salt hydrolase activity and resistance to bile
2. Generation of microbial metabolites from dietary components (for example equol and/or other beneficial aglycones)
3. Reduction of DNA damage. Antigenotoxic properties against chemical carcinogens
4. Glutathione-S-transferase, glutathione, glutathione reductase, glutathione peroxidase, superoxide dismutase, and catalase
5. Butyrate production
<i>Bacterial activities that have been linked to increased risk of colorectal cancer</i>
1. $\beta$ -glucuronidases
2. H <sub>2</sub> S generation
3. Azoreductases
4. Nitroreductases
5. Alcohol dehydrogenases
6. Arylsulfatases
7. ROS generating enzymes

ROS, reactive oxygen species.

compound of interest and its metabolites, and animal experiments can take months to complete and require large quantities of product. New testing tools such as bioassays, computer modeling techniques, biomarkers, and validated surrogate endpoints are required to improve validation of new probiotic strains or combination of strains and to permit earlier termination of ineffective preparations and thereby reduce research costs.

Traditionally mouse intestinal models and genetic intervention to generate knockout mouse models have been the approach of choice to test and validate the physiological properties of probiotics in vivo (Table 1). Increasing evidence that the microbiota help maintain a healthy GI homeostasis has been derived from studies in germ-free rodents. These models have clearly demonstrated that, in the absence of an intestinal microbiota, intestinal motility is disrupted and also that normalization of motor patterns does not occur with all species of the intestinal microbiota. These studies have also introduced the concept of species specificity, demonstrating that effects on gut function obtained with one bacterial species cannot be extrapolated to another (45, 119). Recent transplant experiments using germ-free mouse recipients of microbiota from obese or lean mice demonstrated the clear influence of the microbiome populations on host obesity. This pioneer study confirmed how different energy-harvesting abilities of the adapted microbiota can influence food assimilation by the host (264). Following this approach, we should be able to find microbial populations more adapted to environmental toxins, specific diets, etc.. Novel in vitro models should be able to accurately assess the ADMET steps for new probiotic candidates early in the development process, allowing researchers to reduce costs and ethical concerns when screening characteristics that are host independent and replicable in vitro. In vitro systems cannot fully simulate the physiological processes of the gut wall, such as active or facilitated transport and local cellular and humoral immune system and feedback mechanisms. However, they are essential to better understand the characteristics of individual members of the microbiome as well as the molecular basis of the interaction between microorganisms and to explore practical applications. Consequently, to investigate the intestinal microbiota as an ecosystem, several in vitro systems have been developed. Examples of such models are the single bioreactor GI tract simulator, the Human Intestinal Microbial Ecosystem (SHIME) (180, 222, 254), a three-step colon model (157), a semicontinuous four-channel colon simulator (158, 159), and an in vitro microscale cell culture analog of the GI tract (171). Other in vitro systems designed to study the intestinal microbiota are the multicompartmental, dynamic, computer-controlled systems that simulate the human GI tract, TIM-1 (178), and large intestinal system TIM-2 (28, 178), which were used to investigate specific drug dosage forms for colonic or rectal delivery and the effect of the complex microbiota on the stability of drug compounds.

### Conclusions

The bacteria associated with the human body outnumber our own cells by at least one order of magnitude, and the majority reside within our GI tract. The GI microbiota allows us to defend ourselves from pathogens and digest nutrients, but it is

also implicated in human disease susceptibility including risk of CRC. The rapid evolution of “omics” technologies is beginning to provide researchers the information needed to modulate that immense bacterial ecosystem by introducing a carefully selected blend of beneficial organisms capable of survival, persistence, and delivery of a wide range of bioactive compounds. Certainly, the beneficial effects of probiotics, which are greatly influenced by our individual and varied genetic makeup and environment, including diet, have been long debated because of conflicting research. However, present and future studies will help overcome these barriers to enable development and more rigorous testing of probiotics as more natural and less disruptive treatments to prevent CRC and other GI-related malignancies.

### ACKNOWLEDGMENTS

The authors thank P. K. Lund and E. Altermann for their insightful comments and critical reading of this review.

### DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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