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Microbial Metabolites as Molecular Mediators of Host-Microbe Symbiosis in Colorectal Cancer

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Abstract

The symbiosis between the gut microbiota and the host has been identified as an integral part of normal human physiology and physiological development. Research in germ-free or gnotobiotic animals has demonstrated the importance of this symbiosis in immune, vascular, hepatic, respiratory and metabolic systems. Disruption of the microbiota can also contribute to disease, and the microbiota has been implicated in numerous intestinal and extra-intestinal pathologies including colorectal cancer. Interactions between host and microbiota can occur either directly or indirectly, via microbial-derived metabolites. In this chapter, we focus on two major products of microbial metabolism, short-chain fatty acids and bile acids, and their role in colorectal cancer. Short-chain fatty acids are the products of microbial fermentation of complex carbohydrates and confer protection against cancer risk, while bile acids are compounds which are endogenous to the host, but undergo microbial modification in the large intestine leading to alterations in their bioactivity. Lastly, we discuss the ability of microbial modulation to mediate cancer risk, and the potential to harness this ability as a prophylactic or therapeutic treatment in colorectal cancer.

1 The Gut Microbiota

The human microbiota is a community of bacteria, archaea, protists, fungi, and viruses that live in and on the human body (1). The term gut “microbiome” is sometimes used synonymously with the gut “microbiota” but can also refer to the full collection of genes present in the microbiota of a community. The cells of our microbiota are estimated to outnumber our nucleated human cells by a ratio of about 13:1, about 70% of which occupy our gastrointestinal (GI) tract (2). A symbiotic relationship exists between the microbiota and host, and this relationship plays a vital role in host immune modulation, metabolism,

inhibition of pathogens and structural development (3, 4). Members of the microbiota may be classified by the nature of their symbiotic relationship with the host, ranging from harmful pathogens to beneficial probiotics. These probiotic bacteria are characterised as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host", while prebiotics are "selectively fermented ingredients that result in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health" (5-7). Some relationships are more complex however, with species displaying both harmful and beneficial behaviour. *Helicobacter pylori*, for example, is recognised as a major risk factor for stomach cancer, but the elimination of this species has been associated with increased rates of inflammatory diseases including inflammatory bowel disease (IBD), asthma and eczema, suggesting a role for *H. pylori* in immune modulation (8, 9).

The gut microbiota comprises over 5000 bacterial species and 3 million genes in a typical individual, with possibly over 35,000 species in the collective human microbiome (10, 11). It is dominated by the phyla Firmicutes and Bacteroidetes, featuring smaller proportions of Proteobacteria, Verrucomicrobia, Actinobacteria, Fusobacteria, and Cyanobacteria (3, 12). This consistency of phyla, combined with significant inter-individual variation within the phyla, suggests a selective pressure to maintain the higher taxonomic structure with a functional redundancy at lower levels (12, 13). The upper GI tract contains relatively few microbial inhabitants. The stomach and duodenum contain approximately 10^2 organisms per gram of contents. This rises to 10^4-10^7 in the jejunum, finally reaching ~10^9 colony forming units (CFUs)/mL in the terminal ileum and ~10^{12} CFU/mL of primarily anaerobic bacteria in the colon (3, 14). The composition also changes along the length of the GI tract, with *Bacillus* and Actinobacteria enriched in the small intestine, while Bacteroidetes and *Lachnospiraceae* are enriched in the large intestine (11).

The intestinal tract is generally considered sterile at birth, with colonisation beginning immediately through contact with the mother and environmental bacteria. Recent research, however, has suggested colonisation of the placenta by *Streptococcus agalactiae* in approximately 5% of pregnancies. However, the possibility remains that this is a result of sample contamination (15, 16). The newborn microbiota is reflective of the mode of delivery, with babies delivered by Caesarean section having a microbiota characterised by fewer *Bifidobacterium* species compared to vaginal births (17). The shift towards an adult microbial composition begins during weaning before the microbiota stabilises at approximately 1-2.5 years of age (18). The microbiota then remains largely stable until old age, in the absence of disruptions such as long-term dietary changes or migration (19, 20). Further changes to the microbiota are observed later in life, such as a reduction in diversity and in the number of symbiotic species, and an increase in enteric bacteria, which may be associated with the age-related physiological decline observed in these populations (21-23).

1.1 Host-microbe symbiosis and physiological development

The ancient association and co-evolution between host and microbe have lead to the deep integration of the microbiota into normal physiological processes and development. This is illustrated by germ-free (GF) animals, which, in the absence of normal gut microbiota, display several developmental abnormalities including an immature immune system (24). Potential mechanisms by which the neonatal microbiota mediate the development of the immune system differ between bacterial species, and likely involve the interacting influences of many different taxa. GF mice have a suppressed T Helper Type 1 (T_h1) cell response that can be restored by monocolonisation with *Listeria monocytogenes*, which stimulates interleukin

(IL)-12 production in macrophages. Likewise, the reduction in T_h17 cells observed in these animals can be normalised by colonisation by segmented filamentous bacteria (SFB) leading to the release of serum amyloid A from intestinal epithelial cells (25). Colonisation with SFB also upregulated the production of immunoglobulin A, which is crucial for a tolerance of commensal microbiota by the mucosal immune system (26, 27).

The host immune response also modulates the composition of the gut microbiota, and the ability of the mucosal immune system to differentiate between commensal and pathogenic bacteria is a topic of ongoing research (28). Members of the gut microbiota interact with the host directly by signaling through pathogen recognition receptors, such as Toll-like Receptors (61). The gut microbiota also produces a wide array of bioactive bacteria-derived metabolites, both from compounds endogenous to the host, e.g. bile acids, or exogenous compounds such as those found in the diet or environment, which allow them to interact indirectly with the host. These metabolites can also play an important role in host health and disease, including colorectal cancer (CRC) (discussed in Section 3).

2 Host-Microbiota Interactions in Colorectal Cancer

There is precedence for the involvement of bacteria in GI cancer, as *H. pylori* is the strongest known risk factor for gastric cancer (8). Given the close apposition between the gut microbiome and colonic epithelium, in particular, research efforts have focussed on the role of the microbiota in colon cancer (Table 1) (29). The proposed mechanisms by which the microbiota may impact CRC include its effects on the immune system and proto-oncogenic pathways such as proliferation and apoptosis, while microbial metabolites can also have pro- and anti-tumorigenic associations (30). The strongest links between the microbiota and potentially cancer-promoting inflammation involve pathogenic species such as *Fusobacterium nucleatum* or enterotoxigenic *Bacteroides fragilis*, both of which have been positively correlated with CRC (31, 32). The role of the microbiota in proliferation is evident in GF mice which display smaller intestinal crypts with a lower mitotic index (33), while the microbiota can mediate apoptosis via a number of mechanisms including the production of butyrate (Section 3.1). Moreover, tumour formation is reduced in GF animals (68), with faecal microbial transfer from CRC patients to GF mice increasing tumorigenesis in these animals (69-71). This capacity to regulate both intestinal proliferation and apoptosis highlights the importance of this delicate symbiotic relationship, which could contribute to cell cycle disruption if dysregulated.

[Table 1]

Substantial evidence exists in animal models for the role of gut bacteria in promoting CRC. These studies primarily utilise mouse models either genetically predisposed to CRC such as the APC^{MIN} mouse, or use genotoxic compounds such as azoxymethane (AOM) or its precursor dimethyhydrazine (DMH), to chemically induce CRC. AOM can also be combined with dextran sodium sulphate (DSS) to model colitis-associated CRC. Using this AOM/DSS model of CRC, manipulation of the microbiota with antibiotics was shown to result in reduced tumorigenesis, but antibiotic treatment had conflicting effects in APC^{MIN} mice (63, 64). Antibiotic treatment was protective, however, in APC^{MIN} mice when compound mutations in DNA repair or interleukin receptor genes were present (64-66). Furthermore, Onoue *et al*. observed decreased numbers of aberrant crypt foci (ACF) in DMH-treated GF rats compared to conventional rats (67). Conversely, the administration of bacteria associated with cancer risk, for example, *Streptococcus bovis* or *F. nucleatum*, to susceptible animals was shown to

increase proliferation, inflammation, and tumorigenesis (68, 69). Tumour multiplicity was also increased in gnotobiotic (GB) rats colonised by enterococci compared to GB rats without enterococci, with the tumour numbers in the former group significantly decreased by the inclusion of probiotic strain *Bifidobacterium breve* (67). A similar result was achieved by Horie *et al*. concerning adenomas, with the lowest incidence of adenoma development observed in rats mono-associated with probiotic *Lactobacillus acidophilus* (70).

In contrast, human studies present only associative evidence for the role of the microbiota in CRC. The microbiota is altered in the colon of CRC patients and in the tumour tissue compared to healthy controls, with adenomatous polyps representing an intermediate step between the two states (71). The colonic mucosa is the symbiotic interface between host and microbiota, and studies have shown colonisation of this interface by adherent and invasive *Escherichia coli* in carcinoma patients (58, 72). Moreover, CRC patients had increased carcinogenic microbial metabolites in their faeces compared to healthy individuals despite both groups having similar diets, with the difference ascribed to their different levels of enzymatically-active anaerobic bacteria (73). Similarly, *Lactobacillus* species have been shown to reduce faecal and urinary mutagenicity induced by fried meat consumption and to reduce faecal β-glucuronidase, β-glucosidase, nitroreductase and glycocholic acid hydrolase activity (74-76). The gut microbiota can also modulate the production of mucus in the intestinal lumen, which in itself can play an important role in CRC by regulating the interaction of the gut bacteria and luminal contents with the colonic epithelium (77).

The composition of the microbiota has also been investigated as a potential predictive biomarker for human CRC. Two meta-analyses of human faecal shotgun sequencing studies identified microbial taxonomic signatures with sensitivity to, and specificity for, CRC, which was comparable to common non-invasive clinical screening tests (78, 79). Models based on the functional gene content of the faecal microbiome were also generated, and enrichment of the bile acid-inducible operon, which is involved in microbial bile acid metabolism, was demonstrated at both the genomic and transcriptomic levels (78, 79). Additionally, bacterial species associated with the oral cavity are frequently enriched in gut microbiota in CRC patients, and a model combining data from oral and faecal microbiota was highly predictive of CRC (62).

3 Microbial metabolites as mediators of host-microbe symbiosis in colorectal cancer

Another key interaction between the host and the microbiota is through the production of microbial-derived metabolites (80). Here, we focus on two major products of microbial metabolism, short-chain fatty acids and bile acids, and their role in CRC.

3.1 Short-chain fatty acids

Commensal bacteria contribute to host-microbial homeostasis and resistance to CRC via the production of short-chain fatty acids (SCFAs). SCFAs are fatty acids with less than six carbon atoms and are primarily the product of fermentation of dietary fiber by anaerobic bacteria in the proximal colon (81). The three most common SCFAs are acetate, propionate, and butyrate, with butyrate shown to play a predominant role in CRC (82). The majority of butyrate is produced by bacteria in *Clostridium* clusters XIVa and IV, particularly *Roseburia*/*Eubacterium rectale*-related bacteria in cluster XIVa and *Faecalibacterium prausnitzii* relatives in cluster IV (83). In a screen of butyryl-CoA:acetate CoA-transferase sequences from human faecal samples, 88% of sequences belonged to *E. rectale*, *Roseburia*

faecis, *Eubacterium hallii* and an unnamed species with the remainder coming from uncultured strains (83).

Butyrate is the primary energy source for normal colonic epithelial cells and has been associated with positive health effects, including in CRC (84, 85). Concentrations of SCFAs are highest in the caecum and proximal colon, where the incidence of tumours is low (86). The lowest intracolonic levels of SCFAs are found in the distal colon and rectum, the site of the majority of human CRC. Butyrate was also reduced in a rat model of CRC, where it correlated negatively with tumour mass (87). Moreover, protein feeding increased tumour number in AOM-treated rats which was ameliorated by resistant starch, which is a substrate for microbial butyrate production (88). Mechanisms by which butyrate protect against CRC are presented in Table 2.

Whilst predominantly protective against the development of CRC, butyrate can have pro-tumorigenic effects following CRC onset. One such mechanism involves its ability to act as a histone deacetylase (HDAC) inhibitor *in vivo* where it epigenetically promotes cell proliferation (89). The contrasting effects of butyrate in normal epithelial cells versus CRC cells can be explained by the metabolic fate of intracellular butyrate. The ability to use butyrate as an energy source is lost in malignant colonocytes (67). Instead, these cells perform glycolysis in what is termed the Warburg effect. This causes the accumulation of intracellular butyrate which generates concentrations sufficient to allow butyrate to act as an HDAC inhibitor. This effect in CRC cells is amplified by glucose-induced metabolism of butyrate by ATP citrate lyase to acetyl-CoA, which acts as a histone acetyltransferase in cells exhibiting the Warburg effect (90, 91).

[Table 2]

3.2 Bile acids

Bile acids are endogenous steroid molecules that are conjugated to a glycine or taurine amino acid residue to form bile salts and stored in the gallbladder for post-prandial release into the duodenum to aid lipid digestion. They are derived from cholesterol and are the major route of cholesterol elimination from the body. The major human bile acids are cholic acid (CA) and chenodeoxycholic acid (CDCA), while in mice the majority of CDCA is converted into muricholic acid (MCA) (110). Although most bile salts are reabsorbed in the distal ileum, around 5% escape to the large intestine where they can be modified by intestinal bacteria (111). These bile acids undergo deconjugation of the amino acid residue by *bile salt hydrolase* to form free bile acids, followed by 7α-dehydroxylation to form cytotoxic secondary bile acids, as well as a number of other minor modifications (112). 7α-dehydroxylation of the major human bile acids CA and CDCA forms deoxycholic acid (DCA) and lithocholic acid (LCA) respectively. These modifications can alter the biochemistry and bioactivity of bile acids, as well as their receptor specificities, which affect their role in CRC. The synthesis and microbial metabolism of bile acids are presented in Figure 1.

Secondary bile acids are hydrophobic, cytotoxic molecules and evidence suggests they play a role in CRC. For example, numerous epidemiological studies have highlighted higher faecal bile acid content in populations with increased CRC rates (113-115). Moreover, DCA is higher in patients with colorectal adenomas and was first proposed as a carcinogen in 1940 based on its induction of tumours in mice (116, 117). Bile acids were initially classified as tumour promoters rather than tumour initiators, as studies primarily demonstrated their action when co-administered with chemical carcinogens such as AOM (118, 119). However, the role

of bile acids as aetiologic agents of cancer in their own right is now emerging (120). For example, a diet high in fat and low in fiber is a known risk factor for colon cancer (121). This diet was also associated with increased secondary bile acids, as well as increased glucuronidase deconjugation (121). Also of note, GF rats are generally resistant to chemical carcinogen-induced CRC (122). However, rats treated with the chemical carcinogen methylnitronitrosoguanidine (MNNG) and DCA displayed colonic adenocarcinomas, suggesting microbial production of DCA could play a role in tumorigenesis and may explain, in part, the resistance to CRC observed in GF animals (123).

Bile acids can increase cancer risk by several mechanisms. DCA and CDCA were shown to up-regulate pro-inflammatory cyclooxygenase-2 and its downstream inflammatory product prostaglandin E_2 in a protein kinase C-dependent manner, whilst activating c-Jun and AP-1 (124, 125). Bile acids also generate reactive oxygen and nitrogen species via a detergent effect on cell membranes and activation of inducible nitric oxide synthase (126). Additionally, bile acids may induce apoptosis in the short term but select for apoptosis-resistant cells in the longer term (127). This ability appears to be related to bile acids' hydrophobicity, with the most powerful effect displayed by the most hydrophobic bile acids (128). Indeed, normal cells adjacent to tumour tissue in colon cancer patients were shown to display resistance to bile salt- and bile acid-induced apoptosis, and this is mediated by an up-regulation of the anti-apoptotic protein B-cell lymphoma-extra large (127, 129).

Bile acids can also induce chromosomal abnormalities such as aneuploidy and micronucleus formation (130, 131). In yeast, DCA, LCA, CDCA and CA each induced mitotic chromosome aneuploidy, while tauro- or glyco-conjugated DCA did not (132). Oxidative stress is a well-established source of chromosomal instability and this is a plausible mechanism of bile-acid induced DNA damage and increased CRC risk (133, 134). LCA was also shown to inhibit the repair activity of DNA polymerase β which could exacerbate the consequences of bile acid-induced DNA damage (135). Finally, a proteomic study of CRC cell lines induced with DCA identified alterations in ten proteins involved in DNA repair and cell cycle checkpoints (136).

Bile acids have also been associated with cancer through Farnesoid X Receptor (FXR) signalling (137). Bile acid homeostasis is regulated by FXR, which is a nuclear receptor expressed by liver hepatocytes and small intestine enterocytes (138). *FXR* expression is down-regulated in human colorectal tumours and colon cancer cell lines (139), while $Fxr^{-/-}$ mice are predisposed to multiple cancers, including that of CRC (140, 141). Moreover, administration of tauro-conjugated βMCA, which is an FXR antagonist bile acid, increased stem cell proliferation by activating Wnt signalling, impaired intestinal integrity, accelerated tumour growth, induced dysplastic morphology and chromosome instability, and increased the serum levels of pro-inflammatory cytokines in APC^{MIN} mice (142). FXR agonists, in turn, promoted apoptosis, down-regulated intestinal stem cell genes and inhibited Wnt signalling (143). FXR agonists also delayed tumour progression, reduced tumour multiplicity, proliferation and serum cytokines, and improved intestinal morphology, differentiation, barrier function and bile acid homeostasis (142). Microbial modification of bile acids plays a role in their interaction with FXR, as FXR displays greater affinity for conjugated bile acids, with reducing affinity for CDCA>DCA=LCA>CA (144). As a result, bacterial modification of bile acids can influence their specificity for FXR and hence their influence on cancer risk. FXR has also been demonstrated to modulate the microbiota as FXR antagonism increased the proportion of Bacteroidetes compared to Firmicutes (145, 146). FXR can also suppress expression of pro-inflammatory cytokines (147), to the extent that a synthetic FXR ligand protected mice from DSS-induced colitis (148).

4 Pre- and pro-biotics as modulators of host-microbe symbiosis: implications for colorectal cancer

Clinical trials have provided evidence for the beneficial role of pre- and pro-biotics in CRC (Table 3). One such trial using a combination of pre- and pro-biotics comprising inulin, *Lactobacillus* and *Bifidobacterium* administered to individuals at high risk of CRC development showed that the combination treatment resulted in a decrease in colonic epithelial proliferation, decreased abundance of *Clostridium perfringens* and reduced ability of faecal water to induce necrosis in colon cells *in vitro* (149). Epithelial barrier function, which is deficient in CRC, was also improved (151).

[Figure 1]

This beneficial effect of pre- and pro-biotics has been replicated in several studies (152-155). Moreover, a prebiotic mixture decreased chemotherapy-associated side effects including diarrhoea and enterocolitis in CRC patients (156). Furthermore, the administration of probiotics can have potential cancer preventative effects. For example, a mixture of *Lactobacillus* and *Propionibacterium* administered to healthy subjects reduced faecal levels of the bacterial enzyme β-glucuronidase, which is implicated in the activation of carcinogens in the colon (157).

[Table 3]

In animal studies, *Bifidobacterium longum* has been shown to ameliorate AOM/DMH-induced colon carcinogenesis, an effect that is enhanced by co-administration with the prebiotics inulin and lactulose (175, 176). A similar effect was seen with *Lactobacillus* species, although this effect was absent when probiotic administration was delayed until 9 weeks into DMH-administration, suggesting *Lactobacillus* was only protective in the early stages of tumorigenesis (177, 178).

The ability of probiotics to affect early-stage cancer development could be due to their function as anti-mutagenic agents. For instance, *Lactobacillus casei* gavage attenuated DNA damage induced by MNNG in rat colonic and gastric mucosa, while in another study, a selection of lactic acid bacteria (LAB) inhibited the genotoxic effects of MNNG and DMH in the rat colon (161, 162). Heat treatment eliminated the protective effect of the bacteria in both studies, suggesting that viable bacteria are required for this effect, although the peptidoglycan fraction and whole freeze-dried *L. acidophilus* were also anti-genotoxic. Arimochi *et al*. also demonstrated a reduction in ACF in AOM-treated rats after the administration of *L. acidophilus* and *C. perfringens* (179). In particular, *L. acidophilus* improved DNA repair by DNA methyltransferase. Other potential mechanisms include the ability of LAB to bind dietary mutagens which limits their ability to interact with the colonic epithelium (167, 180). For example, toxic compounds are detoxified by glucuronidation in the liver, but bacterial β-glucuronidase activity may hydrolyse these molecules and liberate carcinogens. The activity of this enzyme was shown to be reduced in AOM- and DMH-treated rats following gavage with the probiotic *B. longum*. This effect was enhanced by co-administration with the prebiotic inulin, possibly as a result of acidification of the intestinal environment and displacement of bacteria expressing β-glucuronidase (181-183).

Probiotic and commensal bacteria, including species that are indigenous to the normal human microbiota, can also provide health benefits by competing with more harmful organisms and preventing them from becoming established in the GI tract (184). LAB have

been shown to inhibit the growth of coliforms in the GI tract and return *E. coli*-infected rats to a normal microbiota composition while reducing β-glucuronidase activity (185). Probiotics can also produce antimicrobial compounds that inhibit enteric pathogens (186, 187).

Chronic inflammation has been shown to promote CRC and this can be ameliorated by probiotic bacteria (188). This can be mediated by the production of anti-inflammatory metabolites such as butyrate (Section 3.1). Some probiotic bacteria have also been shown to suppress the production of inflammatory factors by host immune cells, with *Lactobacillus reuteri* being shown to suppress the production of tumour necrosis factor-alpha (TNFα) and monocyte chemoattractant protein 1 production by lipopolysaccharide-activated monocytes and macrophages (189). A similar anti-inflammatory effect was also observed in rat pups (190). As well as inhibiting pro-tumorigenic inflammation, probiotics may also induce the targeted production of immune-activating cytokines to suppress tumorigenesis. For instance, the *L. casei* strain Shirota, when administered into the intrapleural cavity of tumour-bearing mice, induced the production of interferon gamma, IL-1β, and TNFα, which in turn inhibited tumour growth and increased survival (191).

5 Conclusions

In summary, the gut microbiota is an integral part of normal human physiology. This microbial reservoir of genes and metabolic functions is larger and more dynamic than the human genome, and from this grows a complex symbiosis between microbiota and host. Disruption of this relationship can have widespread negative effects on human health. This chapter has presented evidence of both protective and harmful influences of gut bacteria and their metabolites in CRC, with a particular focus on SCFAs and bile acids. Manipulation of this symbiosis with pre- and pro-biotics has the potential to have considerable health benefits, as we begin to better understand the cross-talk between the gut microbiota and the host in the maintenance of a healthy symbiotic relationship.

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