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The Role and Mechanistic Effects of Gut Microbiota Metabolites in Immunotherapy for Hepatocellular Carcinoma

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Abstract

Immune checkpoint inhibitors (ICIs) have revolutionized the treatment of advanced hepatocellular carcinoma (HCC), but low response rates and drug resistance remain major clinical challenges. Recent data underscore the gut-liver axis as a major determinant of therapeutic outcomes, particularly amid an epidemiological transition towards chronic liver diseases. In this review, we summarize the effects of gut microbiota-derived metabolites on the hepatic tumor immune microenvironment (TIME) and the efficacy of ICIs. Beneficial metabolites such as short-chain fatty acids and primary bile acids promote antitumor immunity by enhancing the infiltration and cytotoxicity of CD8+ T cells, NK cells, and NKT cells. On the contrary, immunosuppressive metabolites such as secondary bile acids and kynurenine promote exhausted TIME, induce M2 polarization of macrophages, and immune evasion. In addition, structural differences between lipopolysaccharides and tryptophan-derived indoles directly modulate toll-like receptor activation and signaling pathways, including NF-κB, PI3K/Akt, and JAK/STAT3. Elucidation of these complex metabolic networks provides critical insights into mechanisms of ICI responsiveness. Collectively, current evidence supports gut-derived metabolites as promising candidates for predictive biomarkers and therapeutic modulation; however, prospective validation, mechanistic confirmation in humans, and standardized microbiome profiling remain necessary before routine clinical implementation.

Keywords: Hepatocellular carcinoma; gut microbiota; microbiota-derived metabolites; tumor immune microenvironment; immune checkpoint inhibitors; gut-liver axis

1. Introduction

1.1 Global Burden of Hepatocellular Carcinoma

According to the latest global cancer statistics, there were approximately 865,269 new liver cancer cases and 757,948 deaths in 2022 (1). Hepatocellular carcinoma (HCC) accounts for over 80% of primary liver cancer cases, making it the most prevalent subtype of liver malignancy (2). The global burden of HCC is substantial, with geographical variation in incidence closely tied to the distribution of risk factors. In East Asia, Southeast Asia, and sub-Saharan Africa, HCC is a major public health problem, mainly due to the high prevalence of chronic hepatitis B virus (HBV) infection (3). For instance, China has approximately 79.7 million HBV carriers, accounting for about 50% of the global total (4), and HBV-related HCC accounts for 60%-70% of all HCC cases in China (5). While viral etiologies (HBV and HCV) historically dominated the HCC landscape, there is a clear epidemiological shift toward metabolic dysfunction-associated steatotic liver disease (MASLD/NAFLD) (6). It is estimated that by 2030, NAFLD will be the leading cause of HCC in the USA (7). Emerging trends indicate a significant rise in NAFLD-related HCC in Western regions, with an estimated 23% increase in NAFLD-driven HCC incidence in Western Europe over the last decade (8). This shift from viral to metabolic etiologies highlights the growing importance of the gut-liver-immune axis, as metabolic dysfunction-associated steatotic liver disease is tightly linked to intestinal dysbiosis and altered metabolic profiles that may directly modulate immunotherapy responses.

The HCC 5-year survival rate has been below 20% worldwide (9), largely due to late diagnosis and limited therapeutic options for advanced HCC. At the time of diagnosis, only about 30% of HCC patients are candidates for curative treatments such as surgical resection, transplantation, or radiofrequency ablation. For advanced or metastatic HCC, systemic therapies are the primary treatment options, yet their efficacy remains far from satisfactory (10). Chronic liver diseases often lead to progressive liver fibrosis, dysregulation of the immune microenvironment, and tumor immune escape, creating a favorable microenvironment for HCC initiation and progression. For example, liver cirrhosis, regardless of etiology, increases the risk of HCC by 10-20-fold compared with the general population (10), and recent studies have shown that gut microbiota dysbiosis promotes progression from cirrhosis to HCC in high-risk populations (11).

1.2 Current Status of Immunotherapy for HCC

The management of non-resectable HCC has been fundamentally altered by the introduction of immune checkpoint inhibitors (ICIs) targeting the PD-1/PD-L1 and CTLA-4 pathways on cytotoxic T cells (12). Clinical approval for these therapies began in 2017, when the FDA authorized nivolumab as a second-line treatment for advanced cases (13), followed by approvals of agents such as pembrolizumab, durvalumab, and atezolizumab (14). A landmark shift occurred with the IMbrave150 trial, which established the combination of atezolizumab and bevacizumab as the preferred first-line intervention. This dual therapy demonstrated a median overall survival (OS) of 19.2 months and an objective response rate (ORR) of 36%, significantly surpassing the 13.4-month median OS observed with sorafenib (15). Furthermore, long-term data indicate that the three-year OS rate for patients with advanced HCC has now reached 25.3% (16).

However, durable responses are achieved only in a subset of patients, and primary or acquired resistance remains a major clinical obstacle (12). Clinical studies consistently report that approximately 60-80% of patients derive limited benefit from current ICI-based regimens, although response rates vary according to treatment combinations, disease stage, and patient selection (17). The mechanisms of ICI resistance in HCC are complex, encompassing tumor cell-intrinsic factors such as PD-L1

overexpression, loss of MHC class I expression, and aberrant activation of oncogenic signaling pathways, as well as tumor microenvironment (TME)-related factors such as immune cell exhaustion, infiltration by immunosuppressive cells, and abnormal cytokine secretion (18). The lack of reliable predictive biomarkers and the complexity of the tumor immune microenvironment (TIME) contribute to the inconsistent efficacy of ICIs, highlighting the urgent need to identify novel modulators of antitumor immunity (19).

In recent years, the gut microbiota has attracted extensive attention as a key regulator of the immune system, and its role in modulating ICI efficacy has become a research hotspot (20). A study concluded that disrupting the gut microbiome with antibiotics (HR: 1.61, 95% CI, 1.44–1.81) or proton pump inhibitors (HR: 1.28, 95% CI, 1.17–1.41) significantly shortened OS in cancer patients receiving ICI therapy, whereas probiotic supplementation was associated with improved treatment outcomes (HR: 0.67, 95% CI, 0.55-0.82) (21), underscoring their clinical significance.

Although these associations are compelling, observational studies cannot fully distinguish microbiome-mediated effects from confounding variables, including antibiotic indication, nutritional status, liver function, and concomitant medications.

1.3 The Gut-Liver Axis and HCC Pathogenesis

The intestinal microbiome is linked to hepatic physiology and immune function through the gut-liver axis, a two-way signaling system facilitated by bile acids (BAs), immune cells, and portal blood flow (22). Because the portal vein provides roughly 70% to 80% of the liver's blood supply, the liver is the primary organ through which materials originating in the gastrointestinal tract pass (23). In a healthy state, the gut microbiota supports immune equilibrium and maintains the integrity of the intestinal lining by releasing beneficial metabolites and regulating epithelial cell activity (24). As illustrated in Figure 1, this protective boundary, comprising the mucus layer, tight junctions, and epithelial cells, is essential for preventing the systemic migration of gut-derived pathogens, antigens, and metabolic byproducts (25).

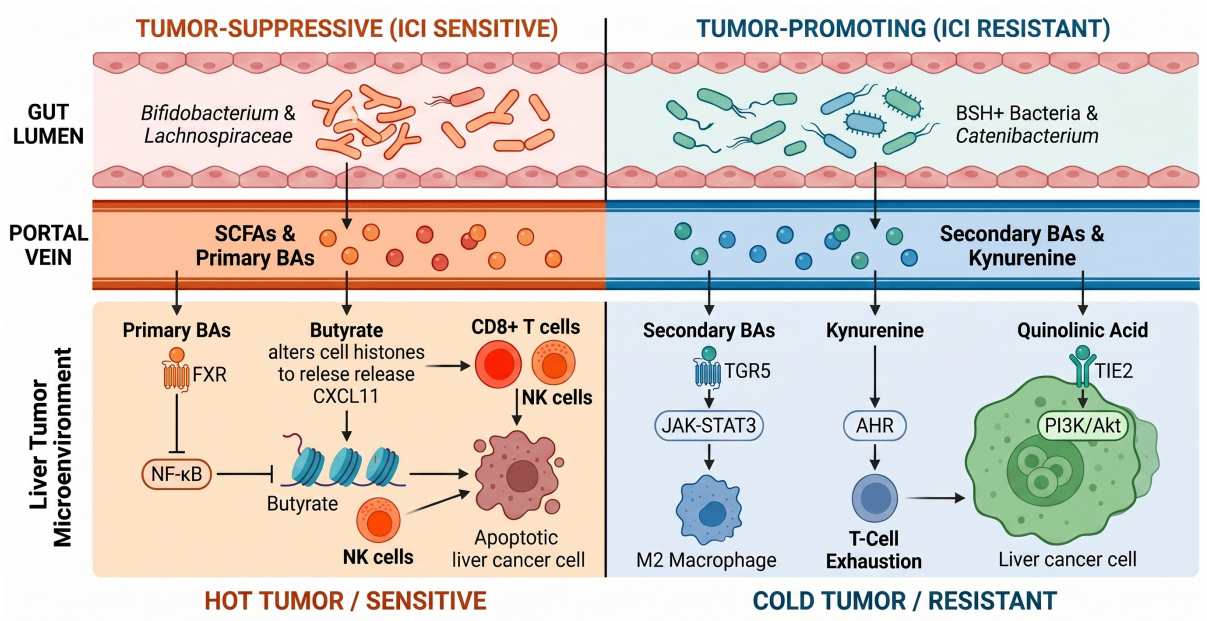


Figure 1. The structural and conceptual framework of the gut-metabolite-host axis dictating immunotherapy efficacy in HCC. *This schematic synthesizes the multi-tiered communication network linking gut microbial ecology to hepatic tumor biology, establishing the gut-liver axis as a critical gatekeeper of ICI responsiveness. By mapping upstream*

microbial composition directly to downstream host signaling cascades, this schematic provides a coherent framework for understanding patient-specific therapeutic variations. This highlights the gut-metabolite-host axis not merely as a correlative feature, but as a functional determinant of ICI resistance and a promising frontier for developing non-invasive predictive biomarkers and microbiome-targeted combination therapies to improve clinical outcomes in advanced HCC.

In chronic liver disease, intestinal dysbiosis leads to intestinal barrier dysfunction (26). For example, HCC patients often show a marked reduction in the abundance of short-chain fatty acid (SCFA)-producing bacteria (e.g., *Lachnospiraceae*, *Ruminococcaceae*) and an increase in pathogenic bacteria (e.g., *Escherichia-Shigella*, *Klebsiella*) (27). This dysbiosis pattern is more pronounced in ICI-resistant patients. Intestinal barrier dysfunction allows microbial metabolites, including LPS, secondary BAs, antigens, and bacteria, to translocate to the liver via the portal vein, thereby triggering hepatic inflammation, fibrosis, and immune dysregulation (24). These gut-derived factors modulate hepatic inflammation by activating pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) on hepatic immune cells (28, 29). For example, LPS binds to TLR4 on Kupffer cells, activating the NF-κB signaling pathway and inducing the production of pro-inflammatory cytokines that promote liver fibrosis and HCC development (30).

Recent data further revealed that *Enterococcus faecalis* in liver tumors promotes HCC progression by delivering Obg GTPase via extracellular vesicles to activate the host mTOR pathway, identifying a direct cross-kingdom mechanism of bacterial-driven carcinogenesis with potential therapeutic implications for mTOR inhibitor treatment in affected patients (31).

Emerging evidence indicates that gut microbiota metabolites are key messengers in gut-liver axis crosstalk, directly shaping the hepatic TIME and ICI responsiveness (32). Ma *et al.* demonstrate that the gut microbiome regulates hepatic antitumor immunity by modulating the balance between primary and secondary BAs, thereby modulating the CXCL16-dependent recruitment of NKT cells to the liver microenvironment (33). These observations suggest that targeting gut microbial metabolism may represent a complementary strategy to improve immunotherapy, although prospective interventional studies remain limited (34, 35).

2. Key Gut Microbiota Metabolites Involved in HCC Immunotherapy

Gut microbiota metabolites, including SCFAs, BAs, lipopolysaccharides (LPS), tryptophan (Trp) metabolites, indole derivatives, and bacterial toxins, are the primary mediators of microbiota-liver-immune crosstalk. Their production is shaped by microbial composition, dietary substrates, host genetics, and environmental factors, and they exert distinct regulatory effects on antitumor immunity and ICI efficacy in HCC. Importantly, metabolite abundance is highly dynamic and may vary according to diet, liver function, medication exposure, geography, and analytical methodology, complicating direct comparisons across studies.

As illustrated in Figure 2, the biological functions and immunomodulatory effects of these key metabolites are crucial for developing microbiota-targeted strategies to enhance HCC immunotherapy. Recent multi-omics studies integrating bulk and single-cell transcriptomics have established a novel polyamine metabolism-related signature (PMRS) comprising 9 genes, which is closely associated with prognosis and immunotherapy sensitivity in HCC, thereby identifying potential biomarkers for personalized treatment strategies (36).

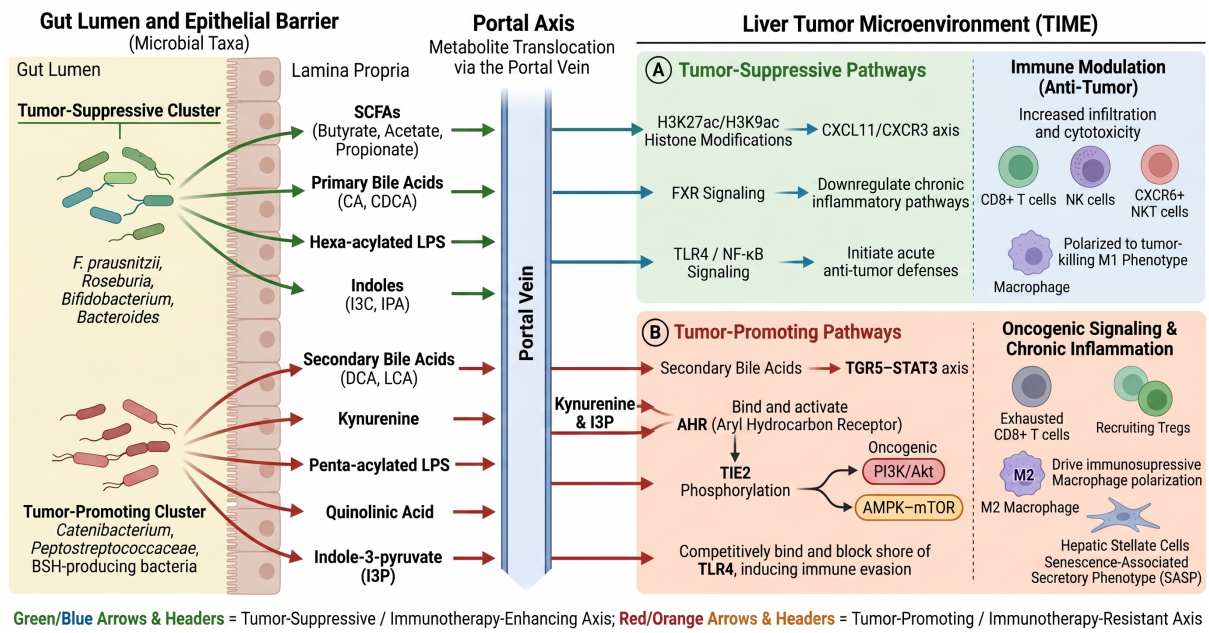


Figure 2. The Synthesized Microbiota-Metabolite-Host Interaction Axis in HCC Immunotherapy. *This schematic framework maps out the unidirectional flow of the gut-liver axis and its dual impact on therapeutic outcomes. The Tumor-Suppressive/ICI-Enhancing Axis is illustrated in green and blue. Beneficial metabolites such as SCFAs, primary BAs, hexa-acylated LPS, and selective indoles target epigenetic machinery, the CXCL11/CXCR3 axis, and FXR signaling, thereby driving favorable immune modulation, including enhanced infiltration of CD8⁺ T cells, NK cells, and NKT cells, alongside antitumor M1 macrophage polarization. The Tumor-Promoting/ICI-Resistant Axis is illustrated in Red/Orange. Immunosuppressive metabolites, including secondary BAs, Kyn, quinolinic acid, and penta-acylated LPS, trigger oncogenic signaling and sustain chronic inflammation. This shapes an exhausted TIME characterized by M2 macrophage polarization, Treg recruitment, and hepatic stellate cell senescence, ultimately driving resistance to ICIs.*

2.1 Short-Chain Fatty Acids

SCFAs are the end products of anaerobic fermentation of dietary fiber by gut bacteria. The three primary SCFAs are acetate, propionate, and butyrate, with acetate the most abundant in the colon, followed by propionate and butyrate in a molar ratio of approximately 60:20:20, though these ratios can vary with diet and microbiota composition (37). The main producers of SCFAs are anaerobic bacteria in the phyla *Firmicutes* and *Bacteroidetes*, including the genera *Ruminococcus*, *Butyricicoccus*, *Roseburia*, *Faecalibacterium*, and *Bacteroides* (38). Once produced, SCFAs are readily absorbed by the colonic epithelium (39). Their metabolic fate, however, differs significantly. Butyrate is preferentially taken up and used as the primary energy source by colonocytes (40). In contrast, acetate and propionate, which are not metabolized in the colon, largely enter the portal circulation and are transported to the liver (41), where propionate serves as a substrate for gluconeogenesis and acetate is used for the synthesis of lipids such as long-chain fatty acids and cholesterol (42, 43).

Multiple independent studies consistently report that HCC patients exhibit significant reductions in fecal and plasma SCFA levels, particularly butyrate, which correlates with poor clinical outcomes and shorter PFS (44). A cross-sectional study of the fecal microbiota in HCC patients found that the abundance of butyrate-producing bacteria, including *Roseburia*, *Eubacterium rectale*, and *Faecalibacterium prausnitzii*, was significantly lower than in healthy controls (45). This reduction in butyrate-producing bacteria is associated with significantly lower plasma levels of acetate, propionate, and butyrate in HCC patients. Furthermore, a recent study showed that in HCC patients receiving immunotherapy, fecal acetate levels were a significant predictor of durable response, PFS, and OS; those with higher acetate levels had significantly longer OS (median: 25.2 vs. 11.3 months; $p < 0.001$)

and PFS (median: 15.3 vs. 4.2 months; $p < 0.001$) (46). These observations support the potential of SCFAs as prognostic biomarkers and therapeutic targets, although prospective validation remains limited.

Butyrate, the most extensively studied SCFA in HCC immunotherapy, has been shown to enhance antitumor immunity by promoting NK cell infiltration and function. Butyrate upregulates CXCL11 expression in HCC cells through epigenetic remodeling, increasing H3K27ac and H3K9ac at the CXCL11 enhancer and recruiting the STAT4 transcription factor, which activates the CXCL11/CXCR3 signaling axis to induce NK cell chemotaxis and cytotoxicity (44). Beyond NK cell modulation, butyrate exerts broad immunoregulatory effects through G protein-coupled receptor signaling (47) and histone deacetylase inhibition (48), influencing the differentiation and function of regulatory T cells (Tregs), effector T cells, and antigen-presenting cells (49). Experimental evidence indicates that butyrate enhances the efficacy of anticancer therapies by directly boosting cytotoxic $CD8^+$ T cell responses through an ID2-dependent mechanism, suggesting potential synergy with immunotherapy (50). The main G protein-coupled receptors (GPCRs) activated by SCFAs include GPR41 (FFAR3), GPR43 (FFAR2), and GPR109A (51). GPR41 and GPR43 are widely expressed on immune cells and intestinal epithelial cells, whereas GPR109A is mainly expressed on dendritic cells, macrophages, and intestinal epithelial cells (52). Activation of these GPCRs by SCFAs can regulate immune cell activation, proliferation, and cytokine secretion, thereby modulating antitumor immunity.

Acetate and propionate also contribute to immune regulation in HCC. Acetate can enhance the cytotoxic activity of $CD8^+$ T cells by regulating cellular metabolism (53). Specifically, acetate derived from *Bacteroides thetaiotaomicron* has been shown to promote M1 macrophage polarization and enhance the cytotoxic function of $CD8^+$ T cells, thereby inhibiting HCC progression and recurrence (42). Propionate inhibits hepatic inflammation by activating PPARα and reducing the secretion of pro-inflammatory cytokines, such as TNF-α (54). Furthermore, propionate has been shown to suppress Ras activity and downregulate PI3K, VEGF, and inflammation-associated pathways, thereby inhibiting cancer cell proliferation and delaying the pathogenesis of HBV-associated HCC (55). Depletion of SCFA-producing bacteria in HCC patients disrupts these protective mechanisms, potentially leading to immune suppression and resistance to ICIs (56).

Whether systemic SCFA supplementation can recapitulate the localized effects observed in experimental models remains uncertain, as circulating concentrations differ substantially from those in the intestinal microenvironment.

2.2 Bile Acids

The composition of BAs, the main metabolites of hepatic cholesterol, is greatly influenced by gut microbiota. BAs undergo enterohepatic circulation, which involves secretion into the intestine and reabsorption by the liver (57). Recent clinical observations have shown a link between changes in the BA profile and HCC incidence, with various BA species exerting distinct and often conflicting effects on immunity and tumor progression (57, 58).

In HCC, primary and secondary BAs generally exhibit opposing immunological effects, although their biological activities remain highly context-dependent and influenced by receptor expression, concentration, and disease stage. The liver synthesizes conjugated primary BAs, including taurocholic acid (TCA), glycocholic acid (GCA), taurochenodeoxycholic acid (TCDCA), and glycochenodeoxycholic acid (GCDCA), by conjugating primary BAs such as cholic acid (CA) and chenodeoxycholic acid (CDCA) with taurine or glycine (57, 59). Conjugated BAs are released into the

intestine, where the gut microbiota, especially bacteria that produce bile salt hydrolase (BSH), deconjugate them and convert them into secondary BAs, including deoxycholic acid (DCA), lithocholic acid (LCA), ursodeoxycholic acid (UDCA), and their conjugated derivatives such as glycodeoxycholic acid (GDCA) (57, 58). The immunological impact of BAs is determined not only by the presence of specific species but also by the homeostatic balance, specifically the ratio of primary to secondary BAs. A prospective cohort study showed that a lower secondary-to-primary BA ratio was associated with increased tumor promotion, whereas higher levels of conjugated PBAs, especially taurine-conjugated CDCA, were associated with a higher risk of HCC (60). Primary BAs such as CDCA promote antitumor environments by facilitating the recruitment of $CXCR6^+$ NKT cells via CXCL16 expression in liver cancer (33). Conversely, an overabundance of secondary BAs can trigger a senescence-associated secretory phenotype (SASP) in hepatic stellate cells, leading to an immunosuppressive milieu (61). The host balances these signals through the bile acid interactome, and a 'tipping point' occurs when gut dysbiosis accelerates the conversion of primary to secondary BAs, effectively switching the hepatic microenvironment from an immune-active to an immune-exhausted state. Monitoring this ratio may ultimately prove more informative than measuring individual bile acids, although standardized clinical thresholds have yet to be established.

The antitumor immunity is regulated by the bile acid-gut microbiota-immune axis. A 2026 study by Xu *et al.* indicates that AF6, a polarity protein, regulates the expression of CYP7A1, the rate-limiting enzyme in bile acid synthesis, thereby serving as a key regulator of primary bile acid synthesis in hepatocytes (62). AF6 knockout increased primary BA levels, which subsequently altered gut microbiota composition and elevated butyrate production, an SCFA. Butyrate, delivered via enterohepatic circulation, upregulated CXCL14 expression and secretion in hepatocytes. This chemokine recruited activated DCs and enhanced $CD8^+$ T-cell cytotoxicity within the TME, thereby creating a tumor-suppressive immune milieu and significantly inhibiting HCC progression. This study established a "BA-metabolism-gut microbiota-immunity" axis as a critical regulator of antitumor immune responses in HCC.

Specific BAs mechanistically modulate immune cell function in HCC through distinct mechanisms. DCA and LCA, two hydrophobic secondary BAs, are associated with the formation of an immunosuppressive tumor microenvironment. DCA suppresses $CD8^+$ T-cell cytotoxicity, promotes M2-like tumor-associated macrophage polarization, and induces hepatic stellate cells to adopt a senescence-associated secretory phenotype, thereby accelerating HCC progression (63). LCA suppresses pro-inflammatory cytokine secretion, promotes M2 polarization via TGR5, and inhibits hepatic NKT cell accumulation by downregulating CXCL16, thereby facilitating tumor immune evasion (64). Lipoteichoic acid (LTA) and DCA can act synergistically to drive macrophage polarization through dual receptor signaling: LTA activates the TLR2-NF-κB pathway to promote M1 activation, while DCA activates the TGR5-STAT3 pathway to promote M2 polarization (65). This "double polarization" creates a unique tumor microenvironment characterized by both pro-inflammatory and immunosuppressive features, paradoxically enhancing HCC progression. The study demonstrated that combined DCA and LTA treatment increased tumor invasiveness and VEGF expression in obesity-associated HCC models. The PNMA1 gene, identified as a novel immune modulator linked to bile acid metabolism, promotes a suppressive TME in HCC upon overexpression, correlating with reduced M1 macrophage and $CD8^+$ T cell infiltration (66). Experimental knockdown of PNMA1 inhibited HCC cell migration and proliferation, indicating that PNMA1 is a potential therapeutic target.

Bile acid-mediated dysbiosis of the gut microbiota further exacerbates immunosuppression. Depletion of beneficial bacteria and enrichment of pro-inflammatory taxa alter the BA pool composition, leading

to accumulation of immunosuppressive secondary BAs and resistance to ICIs. Targeting the bile acid-gut microbiota-immunity axis represents an attractive therapeutic concept that now requires rigorous clinical validation.

2.3 Lipopolysaccharides

Lipopolysaccharide (LPS) is a component of the outer membrane of Gram-negative bacteria. In chronic liver disease, intestinal barrier dysfunction may enhance LPS translocation to the liver via the portal vein, thereby facilitating the pathogenesis and progression of HCC (67). Studies have shown that modulating the gut microbiota to control LPS leakage can inhibit the TLR4/NF-κB pathway and reduce inflammation in HCC. TLR4 is a key component of innate immunity and plays a significant role in the progression of liver disease and tumorigenesis (68). In HCC, TLR4 is functionally expressed and can either promote or inhibit tumor growth, depending on the surrounding cells and environment. In hepatoma cell lines, LPS-mediated TLR4 activation may indirectly facilitate the recruitment of Tregs to the tumor site and augment intrahepatic metastasis through interactions with macrophages (69). A study investigated the effects of LPS on macrophage polarization in the context of HCC (70, 71). The research indicated that LPS at concentrations exceeding 10 ng/mL markedly facilitated M1 polarization of macrophages derived from THP-1 monocytes. LPS treatment redirected the mitochondrial oxidative metabolism of M1 macrophages toward aerobic glycolysis. The study found that LPS-induced M1 macrophages inhibit HCC growth by activating T cells and inducing tumor cell death, which could aid HCC immunotherapy.

New evidence shows that the structure of gut microbiota-derived LPS affects whether immunotherapy works. A groundbreaking study in Nature Microbiology found that patients who responded to anti-PD-1 therapy had more bacterial species with genes for producing hexa-acylated LPS, whereas patients who did not respond had more bacteria producing penta-acylated LPS (72). This difference is important for how things work: hexa-acylated LPS selectively activates TLR4 and NF-κB, prompting macrophages to release cytokines and boosting antitumor immunity. By contrast, penta-acylated LPS blocks immune activation. In murine models of cancer, oral administration of purified hexa-acylated LPS improved the efficacy of anti-PD-1 therapy, leading to enhanced tumor regression and increased infiltration of tumor-specific cytotoxic $CD8^+$ T cells, an effect not observed in mice treated with penta-acylated LPS. Blocking TLR4 with a small-molecule antagonist abolished the beneficial effects of hexa-acylated LPS on anti-PD-1 therapy. These findings support an important role for TLR4 signaling in microbiota-mediated immunotherapy responses, although confirmation in human HCC remains limited.

Modulating the LPS-TLR4 pathway represents a potential treatment strategy for HCC. A study showed that Echinacea purpurea polysaccharide (EPP) treatment in mice with HCC enhanced the proliferation of beneficial gut bacteria that produce butyrate, increased the expression of intestinal tight junction proteins, and repaired the intestinal barrier (67). This reduced LPS leakage, thereby suppressing the TLR4/NF-κB pathway and diminishing the expression of inflammatory mediators such as IL-6 and migratory factors like MMP-2. The research demonstrated that EPP efficiently controlled LPS leakage by altering the gut microbiota, modulating the TLR4/NF-κB pathway, and ultimately hindering HCC-induced tumor cell survival in murine models.

2.4 Tryptophan Metabolites

Tryptophan metabolism in HCC proceeds through three major pathways with distinct immunological consequences: (1) the kynurenine (Kyn) pathway, which is predominantly immunosuppressive; (2) the serotonin pathway, which supports tumor growth and metastasis; and (3) the indole pathway, which

generates both pro-tumorigenic and anti-tumorigenic metabolites depending on the specific derivative and context (73). Understanding how these pathways interact with the gut microbiota is essential for exploiting the antitumor immunity in HCC.

The Kyn pathway promotes immunosuppression and tumor progression in HCC. Indoleamine 2,3-dioxygenase (IDO1, IDO2) and Trp 2,3-dioxygenase (TDO2) are the enzymes that initiate and regulate the Kyn pathway (74). In liver cancer, TDO2 expression correlates with tumor size, stage, differentiation, and recurrence, thereby promoting cell proliferation and tumor growth by regulating p21 and p27 (75). This primary metabolite activates the aryl hydrocarbon receptor, a transcription factor that controls the expression of pro-tumorigenic genes and suppresses antitumor immunity (76). Kyn 3-monooxygenase (KMO) expression is associated with TNM stage, tumor differentiation, and prognosis in HCC patients, and it promotes cell proliferation, migration, and invasion (77). Additionally, 3-hydroxyanthranilic acid (3-HAA), another Kyn pathway metabolite, induces apoptosis in HCC cells through YY1-dependent mechanisms (78).

Serotonin signaling influences HCC progression. It is derived from Trp via Trp hydroxylase and has been implicated in HCC pathogenesis. In HCC patients, elevated levels are associated with worse prognosis, higher Edmondson grade, microvascular invasion, tumor multiplicity, and TNM stage. Serotonin mechanistically promotes cell proliferation, epithelial-mesenchymal transition, and metastasis via the PI3K/AKT/FoxO6 signaling pathway (79).

The indole pathway produces metabolites that play dual roles in HCC. Gut microbiota metabolize dietary Trp into various indole derivatives that exert context-dependent effects on tumor immunity (73). Indole-3-carbinol (I3C), a naturally occurring compound from cruciferous vegetables, has shown substantial immunomodulatory effects in HCC. A study found that I3C prevents PD-L1 from helping HCC evade the immune system by inhibiting the ubiquitination of NF-κB p105 (80). I3C treatment reduced PD-L1 expression in HepG2 cells and increased $CD8^+$ T cell infiltration into tumor tissues. The combination of I3C and anti-PD-L1 monoclonal antibodies produced stronger antitumor effects in mouse HCC models, suggesting it could be used as an immunotherapeutic adjuvant.

On the other hand, indole 3-pyruvate (I3P) acts as an oncometabolite in MYC-driven liver tumorigenesis. A pivotal study demonstrated that MYC-driven liver tumors depend on increased Trp uptake, whereas utilization of Trp to produce Kyn pathway metabolites is diminished (74). Instead, these tumors produce I3P through the activity of interleukin-4-induced 1 (IL4I1). I3P activates AHR, which promotes cell and tumor growth. A No-Trp diet that deprived MYC-driven tumors of Trp not only halted tumor growth but also restored the transcriptional profile of normal liver cells. Conversely, adding I3P to the diet helped Trp-starved cancer cells grow again. These findings identify I3P as a promising therapeutic target in MYC-driven HCC, although the relevance of this pathway across broader HCC subtypes remains to be determined.

New evidence suggests that circulating indole metabolites could help predict how well ICI will work in people with HCC. A study by Jia and colleagues (2024) shows that microbial indole metabolites, such as indole-3-propionic acid (IPA), are important for tumor growth and can serve as adjuvants in tumor immunotherapy (81). Data presented at the American Association for Cancer Research Annual Meeting support this. The data showed that 50% of unresectable HCC patients with high IPA and 10% with low IPA achieved a time-to-progression (TTP) > 8 months (82). TrpNet analysis indicates that the *Peptostreptococcaceae* family is most likely responsible for producing IPA. The *Pasteurellaceae* and *Lachnospiraceae* families, responsible for synthesizing the IPA precursor indole pyruvate, were significantly elevated in individuals who first responded to ICIs. These preliminary clinical

observations suggest that circulating indoles may serve as predictive biomarkers, although validation in independent prospective cohorts remains necessary.

Among the enzymes that gate these pathways, interleukin-4-induced-1 (IL4I1) has emerged as a critical metabolic immune checkpoint. IL4I1 catalyzes the production of indole derivatives and kynurenic acid, which potently activate AHR, thereby promoting tumor cell motility, dampening adaptive immunity, and accelerating tumor progression (83). Notably, IL4I1 expression is induced by immune checkpoint blockade and appears to compensate for IDO1 inhibition, which may explain the disappointing results of clinical trials combining PD-1/PD-L1 inhibitors with IDO1 inhibitors alone. This makes IL4I1 an attractive target for next-generation combination strategies in HCC.

2.5 Other Metabolites

Bacterial toxins, polyamines, and vitamins are additional classes of metabolites derived from the gut microbiota that influence HCC progression and immunotherapy response (84, 85).

Bacterial toxins, particularly LPS from Gram-negative bacteria, contribute to HCC pathogenesis via the gut-liver axis. LPS activates TLR4 signaling, driving chronic inflammation and creating a tumor-permissive microenvironment (84, 85). Dysbiosis-induced intestinal barrier dysfunction increases the translocation of bacterial toxins via the portal vein, generating an inflammatory environment that causes liver damage and may promote hepatocarcinogenesis. Beyond LPS, other bacterial genotoxins, such as cytolethal distending toxin (CDT) produced by certain Enterobacteriaceae species, have been implicated in inducing DNA damage and genomic instability, though their specific role in HCC requires further investigation.

Polyamines, including spermidine and spermine, are produced by gut bacteria such as Bifidobacterium and Lactobacillus and have context-dependent effects on HCC (86). Polyamines can regulate cell proliferation, differentiation, and immune function through multiple mechanisms (87). Recent reviews highlight that polyamines modulate the TIME by influencing T-cell and natural killer (NK) cell function, with potential implications for immunotherapy efficacy (84). Spermidine has been shown to enhance autophagy in immune cells, which may promote antitumor immunity, though clinical data in HCC remain limited. Whether modulation of polyamine metabolism should involve supplementation or inhibition likely depends on tumor context and immune status, highlighting an important area for future investigation.

Gut microbiota-derived vitamins, including vitamins B6, B12, and K, play important roles in regulating antitumor immunity and liver health (84). Vitamin K, particularly vitamin K2, has been studied for its ability to inhibit hepatic inflammation and fibrosis, both of which are critical drivers of HCC progression (88). Protein induced by vitamin K absence or antagonist-II (PIVKA-II) is a well-established clinical biomarker for HCC, though it reflects vitamin K status in tumor tissue rather than direct immunomodulation (89). Vitamin B6 supplementation in HCC patients after resection improved antioxidant capacity primarily by reducing plasma homocysteine, rather than through a direct antioxidant effect (90).

Although these metabolites are presented individually, their biological effects are highly interconnected, and multiple metabolites frequently converge on shared immune and signaling pathways within the hepatic tumor microenvironment. Table 1 summarizes the major gut microbiota-derived metabolites discussed in this review, their principal microbial sources, dominant immunological mechanisms in HCC, and overall impact on ICI efficacy.

Table 1. Gut Microbiota-Derived Metabolites and Their Roles in HCC Immunotherapy

Metabolite Class	Key Metabolite	Major Producing Bacteria / Biological Source	Primary Mechanism in HCC Immunotherapy	Effect on ICI Efficacy
Short-Chain Fatty Acids (SCFAs)	Butyrate	Roseburia, Eubacterium rectale, Faecalibacterium prausnitzii, Butyricicoccus	Enhances NK- and $CD8^+$ T-cell antitumor activity through CXCL11/CXCR3 activation, epigenetic regulation, and NF-κB suppression.	Enhance
	Acetate	Firmicutes and Bacteroidetes (e.g., Bacteroides thetaiotaomicron)	Regulates cellular metabolism to enhance $CD8^+$ T cell cytotoxicity; promotes M1 macrophage polarization to inhibit HCC progression.	Enhance
	Propionate	Anaerobic phyla (Firmicutes, Bacteroidetes, e.g., Ruminococcus, Bacteroides)	Activates PPARα to reduce pro-inflammatory TNF-α; suppresses Ras activity and downregulates PI3K, VEGF, and NF-κB pathways.	Enhance
	Isobutyrate	Bifidobacterium	Stimulates $CD8^+$ T cells, reduces oncogenic JAK/STAT3 signaling pathway activity, and increases IFN-γ secretion.	Enhance
Bile Acids (BAs)	Primary BAs (e.g., CDCA, CA, TCA, GCA)	Host-derived (composition modulated by the gut microbiota)	Promotes NKT-cell recruitment and suppresses NF-κB-mediated inflammation via FXR activation.	Enhance
	Secondary BAs (e.g., DCA, LCA)	Gut microbiota (BSH-producing bacteria)	Promotes M2 macrophage polarization and suppresses $CD8^+$ and NKT-cell antitumor immunity.	Inhibit
Lipopolysaccharides (LPS)	Hexa-acylated LPS	Gut bacteria enriched in anti-PD-1 responders	Activates TLR4/NF-κB signaling to enhance macrophage activation and $CD8^+$ T-cell infiltration.	Enhance
	Penta-acylated LPS	Gut bacteria enriched in anti-PD-1 non-responders	Antagonizes TLR4 signaling and suppresses antitumor immunity.	Inhibit
Tryptophan Metabolites	Kynurenine (Kyn Pathway)	Host and microbial tryptophan metabolism (IDO1/2, TDO2)	Activates AHR signaling to promote immune evasion and tumor progression.	Inhibit
	Indole-3-carbinol (I3C)	Cruciferous vegetables (microbiota-derived)	Suppresses PD-L1 expression through NF-κB inhibition and promotes $CD8^+$ T-cell infiltration.	Enhance
	Indole-3-pyruvate (I3P)	IL4I1-mediated Trp metabolism in MYC-driven tumors	Activates AHR to promote tumor cell growth and motility while actively dampening adaptive immunity.	Enhance
	Indole-3-propionic acid (IPA)	Peptostreptococcaceae (supported by Pasteurellaceae and Lachnospiraceae)	Limits tumor growth and serves as a circulating metabolic driver of tumor immunotherapy response.	Enhance
Other Metabolites	Quinolinic acid	Catenibacterium mitsuokai	Activates TIE2-mediated PI3K-AKT signaling to promote tumor survival.	Inhibit
	Polyamines (e.g., Spermidine, Spermine)	Gut commensals (e.g., Bifidobacterium, Lactobacillus)	Enhances T-cell and NK-cell antitumor function and immune-cell autophagy.	Enhance

3. Mechanisms of Gut Microbiota Metabolites Modulating HCC Immunotherapy Efficacy

Gut microbiota metabolites regulate ICI efficacy by reshaping the TIME and targeting multiple immune cell subsets and signaling pathways (32, 50). The core mechanisms include modulating immune cell activation and function, regulating inflammatory signaling pathways, and influencing tumor cell immune escape (Figure 3). Improved understanding of these pathways may facilitate the development of microbiota-targeted therapeutic strategies (32). Recent single-cell sequencing and spatial metabolomics studies have provided new insights into the spatiotemporal dynamics of these mechanisms within the HCC microenvironment (73).

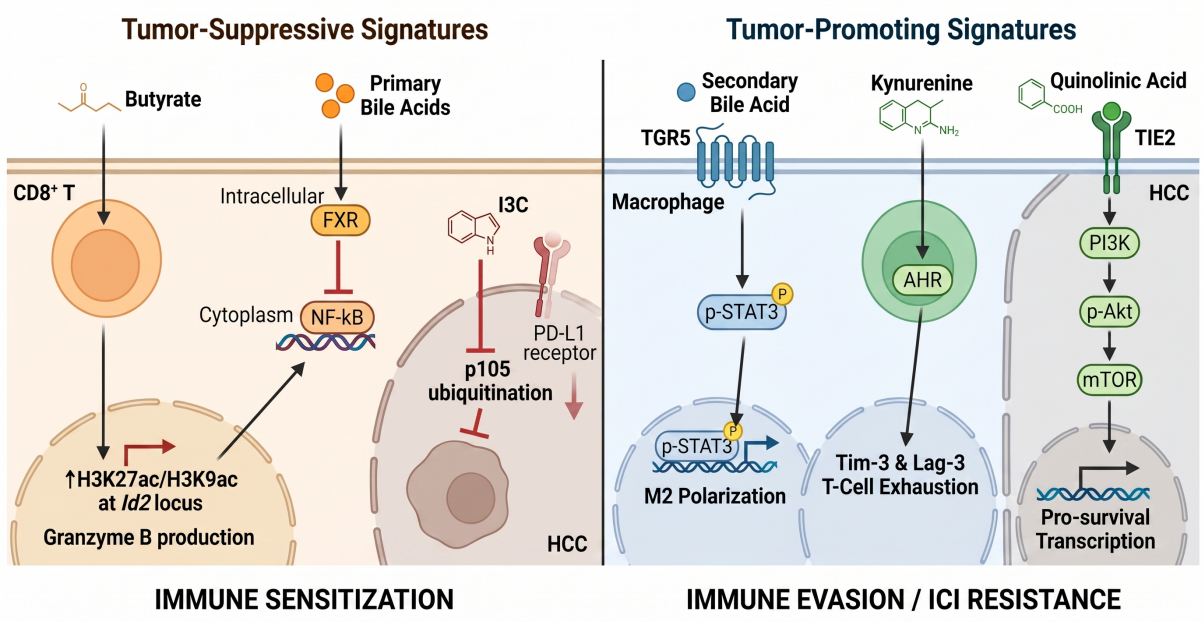


Figure 3. Intracellular signaling pathways by which gut microbiota-derived metabolites modulate immunotherapy efficacy in hepatocellular carcinoma (HCC). *The schematic summarizes how microbial metabolites regulate immune cell function, inflammatory signaling, and tumor cell survival within the hepatic tumor microenvironment. Left (orange/red): Tumor-suppressive pathways. Beneficial microbiota-derived metabolites, including short-chain fatty acids (SCFAs), primary bile acids, and indole-3-carbinol (I3C), enhance antitumor immunity through epigenetic regulation, suppression of NF-κB signaling, promotion of M1 macrophage polarization, recruitment of NK and NKT cells, and reduction of PD-L1 expression, thereby improving responses to immune checkpoint inhibitors (ICIs). Right (blue/green): Tumor-promoting pathways. Dysbiosis-associated metabolites, including secondary bile acids (deoxycholic acid (DCA) and lithocholic acid (LCA)), kynurenine, indole-3-propionic acid (I3P), and quinolinic acid, activate TGR5-, AHR-, and PI3K/Akt/mTOR-dependent signaling networks that promote M2 macrophage polarization, T-cell exhaustion, regulatory T-cell expansion, immune exclusion, tumor-cell survival, and resistance to ICI therapy. Collectively, these pathways illustrate how gut microbiota-derived metabolites remodel the hepatic immune microenvironment by integrating metabolic, epigenetic, inflammatory, and transcriptional programs that determine sensitivity or resistance to cancer immunotherapy.*

3.1 Modulation of Inflammatory and Signaling Pathways

Current evidence indicates that metabolites of the gut microbiota regulate key signaling pathways involved in inflammation and antitumor immunity, including NF-κB, PI3K/Akt, AMPK-mTOR, and FXR/TGR5 (63). These pathways are interconnected, forming a complex regulatory network that modulates immune cell function, tumor cell proliferation, and ICI efficacy.

The NF-κB signaling system is a crucial regulator of inflammation and immune responses. Through engagement of TLR4 and other receptors, LPS and secondary BAs activate the NF-κB pathway, leading

to the synthesis of pro-inflammatory cytokines, including TNF-α, IL-6, and IL-1β (91). Prolonged NF-κB activation may diminish the efficacy of ICI while simultaneously exacerbating hepatic fibrosis, inflammation, and tumor proliferation (92). The endotoxin LPS, originating from the outer membrane of Gram-negative bacteria, binds to the CD14/TLR4/MD-2 receptor complex on innate immune cells. This interaction triggers a MyD88-dependent signaling cascade that activates NF-κB and drives the transcription of pro-inflammatory genes (93). Persistent activation of the LPS-TLR4-NF-κB pathway appears to contribute substantially to chronic inflammation and immune dysregulation in HCC.

NF-κB serves as a crucial signaling hub that integrates multiple pro-tumorigenic signals within the tumor microenvironment. This includes inflammatory cytokines such as TNF-α and microbial detection mediated by TLRs (94). Once in the nucleus, activated NF-κB initiates transcription of target genes, including immune checkpoint molecules that facilitate immune evasion, pro-inflammatory cytokines that sustain chronic inflammation, angiogenic and metastatic effectors, and regulators of proliferation and survival (MYC, BCL2). In contrast, SCFAs such as propionate and butyrate can reduce chronic inflammation by suppressing NF-κB activation (49). A 2024 study demonstrated that Echinacea purpurea polysaccharide (EPP) intervention in HCC models increased beneficial butyrate-producing gut microbiota, enhanced intestinal tight junction protein expression, and repaired the intestinal barrier, which controlled LPS leakage and subsequently inhibited the TLR4/NF-κB pathway, reducing expression of inflammatory factors such as IL-6 and migration factors like MMP-2 (67).

Immune cell survival, proliferation, and polarization are governed by the PI3K/Akt signaling pathway (95). The gut bacterium *Catenibacterium mitsuokai* promotes HCC development by releasing quinolinic acid, which interacts with and activates the TIE2 receptor on HCC cells, as reported (96). Phosphorylated TIE2 then activates the downstream oncogenic PI3K/AKT pathway, accelerating HCC progression. Through its surface protein Gtr1/RagA, which interacts with the γ-catenin receptor, *C. mitsuokai* compromises the gut barrier, translocates to the liver as viable bacteria, and establishes colonization in HCC cells. Its pro-tumorigenic effect relies on the secreted metabolite quinolinic acid. The link between *Catenibacterium mitsuokai* and the PI3K/Akt pathway serves as a template for how microbial metabolites influence hepatic oncogenic signaling. The PI3K/Akt/mTOR axis may serve as a convergent signaling hub for multiple microbiota-derived metabolites, although the relative contributions of individual metabolites remain incompletely defined. For instance, SCFAs can modulate PTEN (a negative regulator of PI3K) via GPCR signaling (97), while high levels of secondary BAs have been shown to activate M2 Macrophages via transactivation of the S1PR2/PI3K/AKT axis (98). This suggests that the microbiota-metabolite axis not only affects immune cells but also directly alters the intrinsic survival signaling of HCC cells, providing multiple potential nodes for therapeutic intervention.

Immune cell activation and function depend on modulation of energy metabolism via the AMPK-mTOR signaling pathway (99). A study demonstrated that the herbal medicine decoction THSWD can slow HCC progression by enhancing the LTF/AMPK/mTOR/Beclin1 pathway and promoting lysosomal autophagy in HCC cells, through regulation of the gut microbiota and metabolic reprogramming. The study found that THSWD increased the metabolite glabrol, which induces apoptosis in HCC cells by activating the lysosomal autophagy-mediated apoptosis signaling pathway, while also increasing the abundance of the intestinal microbiota member *Duncaniella*. Glabrol supplementation enhances lysosomal autophagy and triggers apoptosis in HCC cells by activating the LTF/AMPK/mTOR/Beclin1 pathway.

BAs play a central role in regulating the FXR/TGR5 pathway, which is essential for both immune and metabolic functions (100). Research by Pathak et al. shows that FXR and TGR5 collaborate to regulate

hepatic metabolism and BA synthesis; simultaneous activation of these receptors diminishes inflammation and improves metabolic stability (101). Primary BAs such as CDCA and secondary BAs such as UDCA activate FXR, which inhibits NF-κB pathways and reduces TNF-α and IL-6 levels. Lithocholic acid and deoxycholic acid activate TGR5, resulting in increased IL-10 expression and diminished inflammatory signaling via cAMP pathways. Disruption of the microbiota-bile acid-immune axis often fosters a pro-inflammatory and immunosuppressive tumor microenvironment. This pattern is common in gastrointestinal and hepatobiliary malignancies, aiding tumor immune evasion and progression (101).

The JAK-STAT pathway, a newly identified signaling pathway regulated by metabolites from the gut microbiota, plays a pivotal role in linking cytokine signals to immune cell function and tumor progression (102). Bifidobacterium and its metabolite, isobutyrate, can enhance the anti-PD-1 effect in HCC through the JAK/STAT3 pathway (103). The study indicates that isobutyrate-stimulated $CD8^+$ T cells inhibited liver cancer cells, reduced JAK/STAT3 pathway activity, and enhanced IFN-γ secretion. Bifidobacterium or isobutyrate, in conjunction with anti-PD-1 therapy, led to reductions in tumor size, increases in IFN-γ and $CD8^+$ T cell levels, and decreases in JAK/STAT3 signaling within the tumor microenvironment (104). These findings offer valuable insights into addressing resistance to immunotherapy in HCC by demonstrating that metabolites from the gut microbiota can modulate the JAK-STAT pathway, thereby influencing the equilibrium between pro-tumor and antitumor immune responses (103, 104).

3.2 Regulation of Tumor Cell Immune Escape

Tumor cell immune escape is a key mechanism of ICI resistance in HCC. Gut microbiota metabolites modulate this process by regulating immune checkpoint molecules, MHC expression, and immunosuppressive factors.

Bifidobacterium and its metabolite, isobutyrate, enhance anti-PD-1 efficacy by modulating the JAK/STAT3 pathway. A study demonstrated that isobutyrate-stimulated $CD8^+$ T cells increased IFN-γ secretion, downregulated JAK/STAT3 signaling, and reduced tumor size when combined with anti-PD-1 in mouse models (103). A prospective clinical study of 74 patients with unresectable HCC found that enrichment of *Lachnoclostridium* and depletion of *Prevotella* 9 predicted ICI response, with responders achieving a progression-free survival of 8.8 months versus 1.8 months in non-responders ($p<0.001$) (105).

Secondary BAs promote immunosuppression. DCA, elevated in HCC patients, induces Kupffer cell polarization toward the M2 phenotype via TGR5/FXR receptors, increasing IL-10 and TGF-β secretion (106). This upregulates PD-1 and TIM-3 on $CD8^+$ T cells, contributing to T cell exhaustion. Bile acid metabolism regulates hepatic immune surveillance through NKT cells. Primary BAs enhance CXCL16 expression on liver sinusoidal endothelial cells, attracting $CXCR6^+$ NKT cells and supporting antitumor immunity (33). Secondary BAs reverse this effect, reducing NKT accumulation and impairing immune recognition of liver tumors. CXCL16 expression in human HCC tissue correlates with primary bile acid levels.

A recent study showed that tumor-suppressing multi-enterobacteria (TSME) combined with anti-PD-1 therapy improved outcomes in immunotherapy-resistant HCC (107). In mouse models, TSME plus anti-PD-1 achieved 58.78±7.55% tumor growth inhibition, increasing $CD8^+$ and $CD4^+$ T cells while reducing Tregs in the tumor microenvironment. By contrast, the Kyn-AhR pathway, activated by IDO1-mediated Trp metabolism, suppresses T cell function. Kyn upregulates PD-L1 expression and inhibits $CD8^+$ T

cell IFN-γ production by up to 65±8% (108). LPS from Gram-negative bacteria activates TLR4 signaling on Kupffer cells, recruiting myeloid-derived suppressor cells and promoting pathological inflammation that supports tumor progression (109). Current evidence supports an important contribution of gut microbial metabolites to tumor immune escape; however, the relative importance of individual pathways likely varies among patients and disease etiologies (110).

4. Clinical Evidence Linking Gut Microbiota Metabolites to HCC Immunotherapy Outcomes

Clinical studies have confirmed an association between gut microbiota metabolites and ICI efficacy in HCC patients, identifying specific metabolite signatures that can predict treatment response and guide personalized therapy.

4.1 Metabolite Signatures as Predictive Biomarkers

In a prospective cohort of 102 patients receiving first-line combination immunotherapy (77 V-HCC, 25 MASLD-HCC), etiology-specific microbiota signatures were distinct, but shared metabolomic features were associated with treatment outcomes (111). *Bacteroides ovatus*, *Kluyvera georgiana*, *Klebsiella oxytoca*, and *Enterococcus faecium* were more abundant in MASLD-HCC, whereas *Mediterraneibacter gnavus* ATCC 29149 was notably more common among durable responders. This group also exhibited significantly higher fecal levels of SCFAs, including acetate, propionate, butyrate, and isobutyrate, as well as increased UDCA levels. The predominance of *Bifidobacterium*, along with an SCFA-enriched profile, was associated with a sustained response in very early HCC (V-HCC). Significantly, higher levels of fecal acetate prior to treatment emerged as a robust predictor of sustained response across both etiologies. When stratified by acetate levels, patients exhibited markedly longer OS (median 25.2 months versus 11.3 months; $p<0.001$) and progression-free survival (median 15.3 months versus 4.2 months; $p<0.001$). These findings underscore the potential of acetate as both a biomarker and a therapeutic target. The significance of organic acids as biomarkers is increasingly recognized across oncology. This finding is supported by research in lung cancer, where acetic acid similarly emerged as an independent predictor of survival and immunotherapy response, suggesting that the relevance of this metabolite may extend across cancer types as a systemic regulator of T-cell metabolic fitness. This cross-cancer observation supports the hypothesis that certain gut-derived metabolites maintain a systemic 'immunometabolic tone,' in which acetic acid serves as a crucial substrate for T-cell mitochondrial fitness and memory formation (112).

A comprehensive 2024 study prospectively enrolled 80 patients with advanced HCC receiving ICI treatment (113). The researchers used multi-omics sequencing, incorporating metagenomic, ITS2, and metabolomic data, to identify gut bacteria, fungi, and metabolites associated with clinical outcomes. The study revealed substantial differences in bacteria and metabolites between the durable clinical benefit (DCB) and non-durable benefit (NDB) groups, although fungal variation was less pronounced. A model comprising 18 bacterial species was developed as a predictive biomarker for immunotherapy efficacy, with an area under the curve (AUC) of 75.63%. Additionally, two bacterial species (*Actinomyces sp. ICM47* and *Senegalimassilia anaerobia*) and one metabolite (galanthaminone) were identified as predictive biomarkers for survival in HCC patients undergoing ICI treatment.

Bile acid metabolism in HCC has shown promise as a prognostic marker, as evidenced by multi-omics analysis. A bile acid metabolism signature showed a strong correlation with tumor stage and OS in a study that combined transcriptomic data from 368 patients with HCC and included validation cohorts comprising 221 patients. Significant dysregulation of bile acid metabolism in HCC was evident in the signature, which revealed distinct pathway activations across various clinical subgroups (114). Notable

variations in immune cell infiltration patterns were observed between groups with high and low bile acid metabolism, potentially influencing the TIME. Further reinforcing the notion that bile acid metabolism-related signatures serve as innovative prognostic biomarkers, an additional integrated analysis utilizing single-cell and spatial transcriptomics identified G6PD+ malignant cells exhibiting disrupted bile acid metabolism. These cells are intricately associated with angiogenesis and the development of an immunosuppressive microenvironment.

Indole-3-carbinol (I3C), a naturally occurring compound in cruciferous vegetables, has shown notable immunomodulatory effects in HCC. I3C suppresses NF-κB p105 ubiquitination, thereby preventing PD-L1-mediated immune evasion in HCC (80). I3C treatment increased CD8 T cell infiltration in tumor tissues and reduced PD-L1 expression in HepG2 cells. In mouse models of HCC, combining I3C with anti-PD-L1 monoclonal antibodies produced synergistic antitumor effects, suggesting its potential as a therapeutic adjuvant in immunotherapy. This underscores the importance of Trp-derived metabolites in HCC immunotherapy, despite their therapeutic rather than predictive role.

Altogether, current evidence supports the development of microbiota-derived metabolites as non-invasive biomarker candidates, although standardization of metabolomic platforms and validation in multicenter cohorts remain major priorities.

4.2 Clinical Trials of Microbiota-Targeted Therapeutic Strategies

Preclinical evidence supports several clinical trials investigating microbiota-targeted strategies to enhance immunotherapy efficacy in HCC. These approaches include FMT, probiotic supplementation, and dietary interventions. The FLORA trial (NCT05690048) is a multicenter, randomized, placebo-controlled, double-blind phase II study examining fecal microbiota transplantation (FMT) in conjunction with atezolizumab and bevacizumab for advanced HCC (115). The trial posits that HCC is a particularly appealing cancer type for FMT due to the anatomical proximity and physiological significance of the gut-liver axis. Furthermore, research indicates that FMT may enhance hepatic function in individuals with liver cirrhosis, potentially leading to improved outcomes in this demographic. The primary outcomes are the infiltration of tumoral $CD8^+$ T cells after two treatment cycles and the safety of the combination therapy. Secondary goals include progression-free survival (PFS), OS, and changes in liver function.

The FAB-HCC pilot study (NCT05750030) is a phase II, single-center, single-arm study evaluating fecal microbiota transplantation (FMT) in individuals with advanced HCC who have not responded to atezolizumab and bevacizumab (116). Twelve patients will participate. They will undergo a single FMT via colonoscopy, with donor material from either healthy donors or HCC patients who have responded to PD-(L)1-based immunotherapy. After the transplant, participants will continue receiving atezolizumab and bevacizumab every three weeks. The frequency and severity of treatment-related adverse events are the primary safety measures. Secondary endpoints include optimal radiological response, assessed by RECISTv1.1 and mRECIST, as well as additional exploratory analyses of gut microbiota composition, circulating immune cells, and serum and stool metabolomic signatures. The findings will help clarify FMT's potential to address immunotherapy resistance in HCC. Important unresolved questions include donor selection, microbiome durability, optimal transplantation schedules, safety in cirrhotic patients, and reproducibility across geographically distinct populations.

The role of *Bifidobacterium longum* in improving postoperative liver function recovery in patients with HCC was investigated in a recent clinical trial (NCT04303286, NCT05178524) (117). The study found that patients with delayed recovery of liver function after hepatectomy had notably lower B. longum

levels in both retrospective and prospective cohorts. In the trial, oral administration of a probiotic cocktail containing B. longum reduced delayed recovery rates in surgical patients with HCC, shortened hospital stays, and improved overall 1-year survival. These benefits were linked to changes in key metabolic pathways, including 5-hydroxytryptamine, secondary BAs, and SCFAs. Additionally, the trial observed reduced liver inflammation and fibrosis, as well as increased hepatocyte proliferation. This trial provides evidence that targeted probiotic supplementation can influence clinically significant outcomes in HCC by altering the gut microbiota, despite focusing on surgical rather than immunotherapy outcomes.

5. Discussion

5.1 Challenges and Future Directions

A major limitation in the current literature is the predominance of associative human studies alongside mechanistic animal models, making it difficult to establish causal relationships in patients. The biological complexity of the gut-liver axis means metabolites often show concentration-dependent dual effects. Butyrate maintains Treg function at physiological concentrations but loses this effect at lower levels. Establishing causality remains difficult because of high inter-individual variability influenced by diet, medications, and genetics. Clinical heterogeneity across HCC etiologies further complicates biomarker development, as evidenced by distinct microbial profiles between viral- and MASLD-related HCC despite shared metabolic signatures.

The Chinese Society of Hepatology has published the "Expert Consensus on the Clinical Application of Gut Microbiota Transplant Therapy in Chronic Liver Disease (118), which provides comprehensive guidance on indications, contraindications, donor selection, transplant routes, and safety considerations for FMT in chronic liver diseases, including liver cancer (6). The consensus underscores the growing recognition of microbiota-based therapies as a legitimate clinical approach and provides a framework for standardizing FMT applications in hepatology (6).

In addition to biological complexity, several technical and methodological challenges hinder the translation of microbiota-derived metabolite findings into routine practice. Accurately quantifying metabolite concentrations *in situ* within the hepatic tumor microenvironment is difficult because stool and plasma measurements may not capture local gradients across the sinusoidal and intratumoral niches, and multi-omics integration remains limited by batch effects and variable analytical platforms. Furthermore, perturbations targeting a single metabolite often reshape the broader microbial community, making it difficult to disentangle direct metabolite-mediated effects from secondary ecological changes and underscoring the need for carefully controlled longitudinal and mechanistic studies. Differences in sequencing platforms, metabolomic methodologies, dietary assessment, sample processing, and definitions of treatment response further complicate cross-study comparisons and limit the reproducibility of microbiota-based biomarkers. Greater methodological standardization will be essential for meaningful validation across independent cohorts.

Future research should prioritize prospective validation of metabolite biomarkers in large multicenter cohorts. Fecal acetate has emerged as a promising pan-etiological predictor, and multi-omics integration of metagenomics, metabolomics, and transcriptomics has identified robust functional biomarkers beyond taxonomic composition. Emerging therapeutic strategies include FMT to overcome ICI resistance, probiotic supplementation, and dietary interventions to enhance SCFA production. Ongoing clinical trials will provide crucial safety and efficacy data. Future studies integrating longitudinal microbiome profiling with spatial transcriptomics, metabolomics, and functional validation in clinically

annotated cohorts will be important for distinguishing causal mechanisms from associative signatures and for developing robust, clinically actionable microbiota-based biomarkers and therapeutic strategies. Potential clinically targeted mechanisms are illustrated in Figure 4.

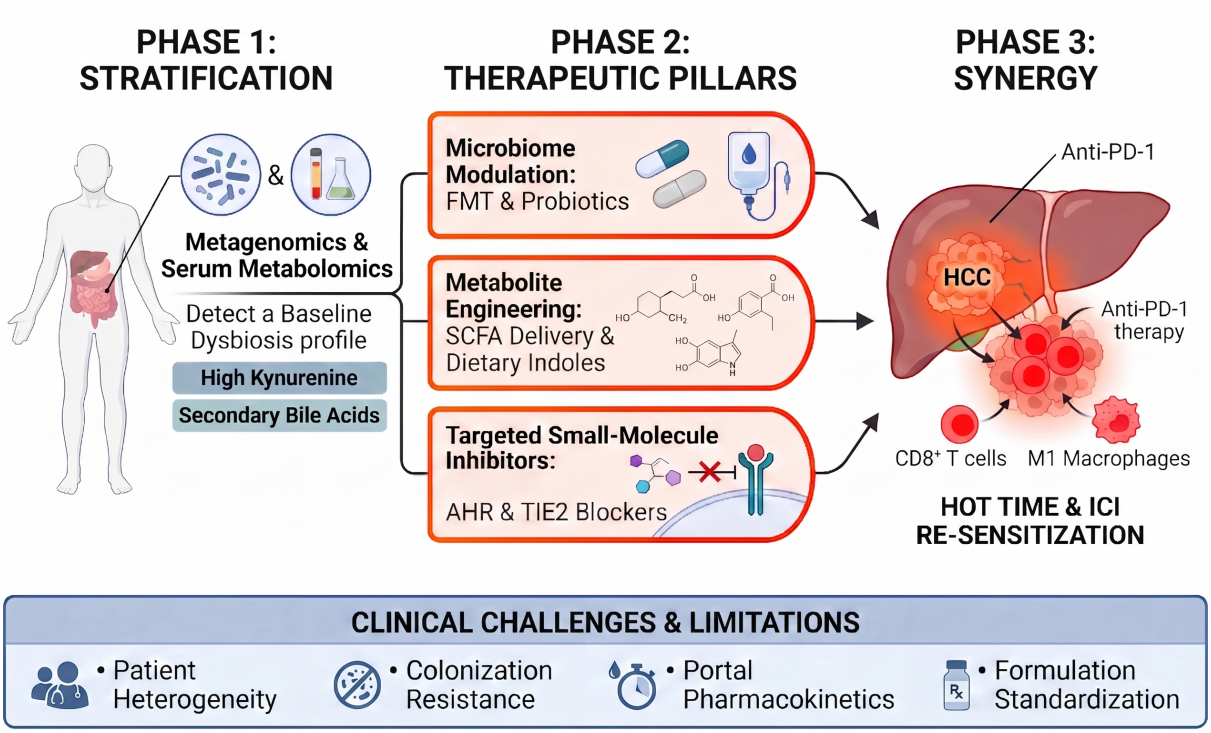


Figure 4. Translational and therapeutic framework targeting the gut-liver-metabolite axis to overcome immune checkpoint inhibitor resistance in HCC. *This schematic outlines the clinical pipeline for translating mechanistic insights into microbial therapeutics, along with major translational hurdles. Multi-omic profiling via metagenomic sequencing and fecal/serum metabolomics identifies baseline dysbiotic signatures indicative of intrinsic ICI resistance. Distinct clinical strategies include microbiome modulation via FMT; metabolite engineering, including targeted SCFA supplementation and tryptophan-indole dietary adjustments; and targeted pharmacological inhibition of immunosuppressive/oncogenic axes. These interventions converge to reshape the hepatic TIME, driving the transition from a "cold," immune-excluded state to a "hot" tumor state characterized by enhanced $CD8^+$ T- and NKT-cell infiltration, M1 macrophage polarization, and re-sensitization to anti-PD-1/anti-PD-L1 therapy.*

Beyond conventional FMT and probiotic supplementation, next-generation strategies are likely to rely on engineered probiotics and synthetic biology platforms that deliver defined metabolites directly to the gut-liver axis, such as butyrate, specific primary BAs, or I3C-like indole derivatives, in a controlled and sustained manner. Rational combination approaches may further enhance efficacy, for example by pairing FMT with high-fiber or resistant-starch diets to boost endogenous SCFA production, or by combining metabolite-targeted agents with PD-1/PD-L1 blockade to concurrently reprogram both the microbiota and the tumor immune microenvironment. Future trials will need to integrate longitudinal multi-omics profiling and adaptive designs to identify which patients benefit most from specific microbiota-modulating strategies and to mitigate the risks of off-target ecological effects.

5.2 Conclusion

Gut microbiota metabolites are critical determinants of immunotherapy efficacy in HCC, serving as key messengers along the gut-liver axis that shape hepatic antitumor immunity. SCFAs, BAs, LPS, and Trp metabolites modulate ICI responses through distinct mechanisms, including immune cell recruitment, epigenetic reprogramming, and regulation of signaling pathways. Clinical evidence identifies fecal acetate as a promising pan-etiological biomarker for patient stratification, while ongoing trials are

evaluating fecal microbiota transplantation and probiotic supplementation to overcome treatment resistance. Despite challenges, including biological complexity and inter-individual variability, microbiota-targeted approaches represent a transformative frontier in HCC immunotherapy. Future research must prioritize prospective biomarker validation and rational combination strategies to translate these mechanistic insights into personalized therapeutic interventions that improve outcomes for patients with this devastating malignancy.

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Declarations

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