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REVIEW

Gut associated metabolites and their roles in *Clostridioides difficile* pathogenesis

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ABSTRACT

The nosocomial pathogen *Clostridioides difficile* is a burden to the healthcare system. Gut microbiome disruption, most commonly by broad-spectrum antibiotic treatment, is well established to generate a state that is susceptible to CDI. A variety of metabolites produced by the host and/or gut microbiota have been shown to interact with *C. difficile*. Certain bile acids promote/inhibit germination while other cholesterol-derived compounds and amino acids used in the Stickland metabolic pathway affect growth and CDI colonization. Short chain fatty acids maintain intestinal barrier integrity and a myriad of other metabolic compounds are used as nutritional sources or used by *C. difficile* to inhibit or outcompete other bacteria in the gut. As the move toward non-antibiotic CDI treatment takes place, a deeper understanding of interactions between *C. difficile* and the host's gut microbiome and metabolites becomes more relevant.

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Introduction

Clostridioides difficile is a Gram-positive, pathogenic, spore forming, anaerobic bacterium that is considered the main cause of antibiotic-associated diarrhea, pseudomembranous colitis and toxic megacolon.[1] According to the Centers for Disease Control and Prevention (CDC), *C. difficile* is a major nosocomial pathogen with more than 220,000 infections, 13,000 deaths, and nearly $5 billion in annual treatment associated costs that are predicted to increase in the future.[2,3] This, combined with its inherent natural antibiotic resistance, led to the CDC classifying *C. difficile* as an 'urgent threat' to the United States healthcare system. The greatest risk factor for *C. difficile* infection (CDI) is prior treatment with broad-spectrum antibiotics. Antibiotics render the host susceptible to CDI by changing the ecology of the microbiota, which is known to provide 'colonization resistance' against invading pathogens, *C. difficile*-included.[4,5] Interestingly, infants and newborns can be *C. difficile* carriers[6] and *C. difficile* can be found in approximately 53% of healthy adults.[7] Thus, *C. difficile* could be considered a part of the microbiome in humans, but this is mostly defined by age.[8]

Endospores are metabolically dormant forms of spore-forming bacteria produced in response to stress (*e.g.*, nutrient limitation).[9–11] For *C. difficile*, the spore form is essential for host-to-host transmission due to the strictly anaerobic nature of the vegetative form.[12–14] Thus, in susceptible hosts, *C. difficile* spores must germinate into the active, vegetative form in order to multiply and cause disease. Germination by *C. difficile* spores is triggered upon recognition of certain bile acids and amino acids by germinant receptors.[11,15,16]

Dormant *C. difficile* spores are considered infectious agents, but the growing *C. difficile* vegetative cells secrete the TcdA and TcdB toxins that are responsible for the primary symptoms of disease (*e.g.*, diarrhea and colitis);[14,17–19] while some *C. difficile* strains secrete a third toxin, a binary toxin.[20] The most common treatments for CDI are broad-spectrum antibiotics like vancomycin, although fidaxomicin (a narrower-spectrum antibiotic)is now recommended for initial and recurrent CDI . Unfortunately, the higher cost of fidaxomicin is limiting its use, and the continued alteration to the colonic microbiota by these antibiotics, lead to patients experiencing recurring disease due to the presence of *C. difficile* spores within the gastrointestinal tract or in the surrounding environment.[21,22]

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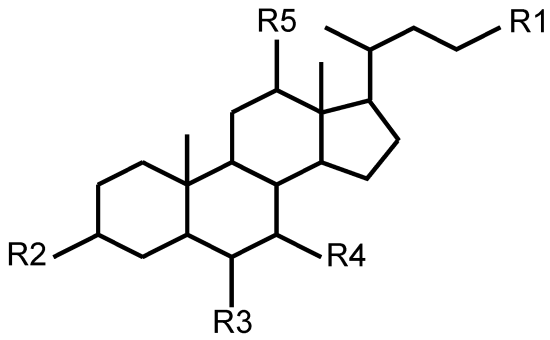
Recently, fecal microbial transplantation (FMT) has emerged as an effective treatment against *C. difficile*, especially for patients with recurrent CDI. FMT is hypothesized to drive protection against CDI through the restoration of gut microbes, gut associated metabolites [*e.g.*, secondary bile acids and short chain fatty acids (SCFA)][23] and/or microbial bile acid biotransformation . Nevertheless, the use of FMT is not without risk for the potential introduction of harmful microbes to the recipient.[24–26] Thus, effective and safe treatments for CDI that result in the restoration of the protective metabolic effects provided by the microbiome still need to be developed. In this review, we summarize studies focusing on metabolites produced by the host or gut microbiota and how these molecules influence *C. difficile* pathogenesis.

Bile acid metabolites and their role in *C. difficile* pathogenesis

Bile acids are cholesterol-based molecules synthesized by hepatocytes in the liver that play key roles in regulating metabolic pathways and aiding in the absorption of fats and cholesterol during digestion.[27,28] There are two pathways for bile acid synthesis: the classical pathway generates both cholic acid (CA) and chenodeoxycholic acid (CDCA) while the alternative bile acid synthesis pathway only forms CDCA.[29] Before being secreted into the intestines, primary bile acids are conjugated with either taurine or glycine at C-24 to generate taurocholic acid (TA)/taurochenodeoxycholic acid (TCDCA) and glycocholic (GCA) acid/glycochenodeoxycholic acid (GCDCA) (Figure 1).[30,31] Reabsorption of bile acids takes place throughout the intestines and the reabsorbed bile acids are recycled back to the liver for additional rounds of digestion.[29,31] During this enterohepatic recirculation pathway, bile acid synthesis is regulated through the Farsenoid X receptor (FXR) transcriptional activator[32] . Bile acid binding principally through CDCA to FXR generates a conformational change in FXR that leads to the synthesis of FGF19 in illeal enterocytes[32,33] . FGF19 is secreted by the ileal enterocytes and binds to the FGFR4 receptor on the cell surface of hepatocytes. Binding of FGF19 to the receptor leads to a negative feedback regulation on bile acid synthesis.[32,33] In an FMT model for recurrent CDI patients, levels of FGF19 were significantly increased post-FMT, thus suggesting upregulation of the FXR-FGF pathway after FMT that may aid in *C. difficile* clearance.[34] Furthermore administration of obeticholic acid, an FXR agonist, to HFD CDI-infected mice resulted in decreases in disease severity, and the use of ursodeoxycholate (UCA) in a CDI mouse model showed increased levels of FXR as well as TGR5 that modulates the immune response against *C. difficile*.[35,36] The mentioned studies on FXR suggest that FXR modulation may be important for CDI treatment but further research is necessary to move past the correlation observed in FXR that shows protection against CDI.

A small percentahe of bile acids escape enterohepatic recirculation, and can be deconjugated by the bile salt hydrolases that are expressed in many gut microbes. Subsequently, deconjugated bile acids can be 7α-dehydroxylated by a small subset of gut microbial species to generate secondary bile acids (deoxycholic acid (DCA), from CA, and lithocholic acid (LCA) from CDCA) (Figure 1).

Cholic acid-derived bile acids promote *C. difficile* germination, whereas bile acids of the CDCA family act as inhibitors of C. *difficile* germination.[16,37,38] In addition to acting as activators/inhibitors of *C. difficile* spore germination, bile acids affect *C. difficile* vegetative growth. The host-derived, primary, bile acids TA and CDCA have different effects on *C. difficile* growth. Vegetative cell growth seems to be unaffected by TA, however *C. difficile* growth is strongly inhibited by CDCA.[16] Interestingly, DCA, a product of the 7α-dehydroxylation of CA by the colonic microbiota, is also toxic to *C. difficile* growth in-vitro, suggesting that the 7α-dehydroxylation of CA by the colonic microbiota may prevent *C. difficile* from colonizing healthy hosts.[16] When the minimal inhibitory concentrations (MIC) of various bile acids were determined, the MIC of CA was 10 mM for 2 different *C. difficile* strains, a concentration that is not found in the GI tract, thus not physiologically relevant.[39] However, increasing the hydrophobicity of the base CA structure (which has three hydroxyl groups, Figure 1), by removing either the 12α hydroxyl (generating CDCA) or the 7α hydroxyl (generating DCA) resulted in the MIC decreasing from 10-fold to 1 mM.[39] Moreover, the 7β-epimer



	R - group					
Bile Acid	**Abbreviation**	**R1**	**R2**	**R3**	**R4**	**R5**
Taurocholic Acid	TA	Taurine	αOH	-	αOH	αOH
Glycocholic Acid	GCA	Glycine	αOH	-	αOH	αOH
Cholic Acid	CA	COOH	αOH	-	αOH	αOH
Deoxycholic Acid	DCA	COOH	αOH	-	-	αOH
Isodeoxycholic Acid	iso-DCA	COOH	-	-	αOH	αOH
Taurochenodeoxycholic Acid	TCDCA	Taurine	αOH	-	αOH	-
Glycochenodeoxycholic Acid	GCDCA	Glycine	αOH	-	αOH	-
Chenodeoxycholic Acid	CDCA	COOH	αOH	-	αOH	-
Lithocholic Acid	LCA	COOH	αOH	-	-	-
Isolithocholic Acid	iso-LCA	COOH	βOH	-	-	-
Hyodeoxycholic acid	HDCA	COOH	αOH	αOH	-	-
Ursodeoxycholic Acid	UDCA	COOH	αOH	-	βOH	-
α - Muricholic Acid	α- MCA	COOH	αOH	βOH	αOH	-
β - Muricholic Acid	B-MCA	COOH	αOH	βOH	βOH	-
ω - Muricholic Acid	ω-MCA	COOH	αOH	αOH	βOH	-

Figure 1. Structures of the bile acids discussed in this review. Shaded rows indicate secondary bile acids. Unshaded rows indicate conjugated and deconjugated primary bile acids.

of CDCA, UCA) (Figure 1), inhibits growth of *C. difficile* cells,[40] although over a 24-h time period, *C. difficile* grew in the presence of 2 mM UCA.[41] Because CDCA completely inhibited the growth of *C. difficile* vegetative cells over a 24-h period and UCA did not, this could imply that stereochemistry of the bile acid structure plays a role in toxicity.[39,40] Importantly, though these two studies were done using different *C. difficile* isolates, which may have different MIC values for bile acids as observed for other *C. difficile* strains.[39]

Mice are one of the most common animal models used in CDI studies and their murine bile acid composition and their effects on CDI are well understood.[39,42] In rodents, the presence of alternative hydroxylating enzymes yields other primary bile acids (α/β/ω-muricholic acids) (Figure 1).[43] Muricholic acids have a MIC similar to that of CDCA and DCA.[39] Similar to CA, muricholic acids are also trihydroxyl bile acids but the hydroxyls are in the 3, 6, and 7 positions instead of the 3, 7, and 12 positions that are found on CA-derived bile acids (Figure 1).[39] A study found that in addition to the bile acids described above, isodeoxycholic acid (a 7α, 12α-hydroxy bile acid), lithocholic acid (a 3α-hydroxy bile acid), isolithocholic acid (a 3β-hydroxy bile acid), and hyodeoxycholic acid also inhibit the growth of *C. difficile* vegetative cells.[44] Furthermore, isoallolithocholic acid (isoalloLCA), the isomer of isoLCA had an

MIC_{90} of 2 μM in *C. difficile* CD630 (a laboratory strain) and in the highly toxigenic *C. difficile* VPI10463 strain.[45]

Due to the inherent toxicity of secondary bile acids for *C. difficile* growth, it was hypothesized that their production would toxify an environment and limit *C. difficile* growth.[16,46] To test this hypothesis, Theriot and Young[5] showed evidence that the presence of secondary bile acids is responsible for protection against CDI. Using a multi-omics approach, the authors identified *C. difficile* resistant and susceptible states in an antibiotic-treated murine model. A susceptible state correlated with high levels of conjugated primary bile acids (*e.g.*, TA) whereas a resistance state correlated with higher levels of secondary bile acids (*e.g.*, DCA). Later, Buffie et al.[4] also provided compelling data in support of this hypothesis. Using mouse models and human subjects, the authors found that high levels of DCA or the presence of the genetic operon that is known to generate secondary bile acids (*bai*), strongly correlated with a protective environment. Additionally, mice treated with different antibiotics resulted in variable metabolic profiles and certain secondary bile acids (*i.e.*, omega-muricholic acid, LCA and, to a lesser extent, hyodeoxycholic acid, and UCA) that could inhibit TA- and DCA-mediated colony formation by *C. difficile* spores.[16,38,39,47]

These landmark studies demonstrated that increased secondary bile acids and the genes encoding proteins responsible for their production strongly correlate with disease-resistant states and increased primary bile acids correlate with disease-susceptible states. A more recent article[45] analyzing metabolites in centennials collected stool from centennials(~107 year olds), elderly adults (85–89 years old), and young adults (21–55 years old) and bile acids were measured. The authors observed higher levels of isoLCA, 3-oxoLCA, alloLCA, 3-oxoalloLCA, and isoalloLCA in the centennial samples, when compared to the other study population. Additionally, the authors characterized the biosynthetic pathways to generate these CDCA derivatives and suggested that further elucidation of microbial biotransformations may expand our understanding of intestinal homeostasis and, as a result, possible protection effects of additional bile acids against CDI[45]

Interestingly, a recent study using a cholate-deficient mouse strain showed that the cholate-derived secondary bile acid (DCA) is dispensable for protection against CDI, and that lack of the bile acid transcriptional activator FXR, does not show differences in CDI mice. Also, when germ free mice were monoassociated with bacteria known to generate secondary bile acids, no secondary bile acids were present in fecal samples, but the mice were still protected against CDI.[48] This emerging evidence would suggest that although bile acids are important for *C. difficile* colonization to be established in the gut, these metabolites may not play such an important role in generating a protective environment against *C. difficile*. In support of this, a 2018 study[49] showed that the levels of the conjugated primary bile acids TA and TCDCA decreased 2 days post-FMT. In contrast, there was an increase of the secondary bile acids DCA, LCA, and UDCA.[49] Interestingly, the authors note that although levels of secondary bile acids increased, the presence of secondary bile acids was not indicative of FMT success or failure, thus suggesting that although bile acids may play a role in protection, there are other mechanisms driving CDI resistance.[49,50]

Regardless, *C. difficile* encounters bile acid during colonization and its growth in the gut may impact bile acid synthesis in the liver. Taking advantage of MALDI imaging mass spectrometry, Wexler et al.[51] found the levels of primary bile acids in the mouse gut increased significantly as early as one-day post-CDI. Additionally, the authors found that the introduction of cholestyramine, a bile-acid sequestering drug, lead to delayed *C. difficile* colonization.[51] Their results suggest that primary bile acids are required to efficiently establish *C. difficile* in a host.

Regarding secondary bile acids, in addition to its toxic effect against *C. difficile* vegetative cells, the secondary bile salt DCA induces *C. difficile* biofilm formation at low concentrations (240 μM) in a non-biofilm-producing *C. difficile* CD630Δerm strain.[52] Thus, it is possible that a decrease in bile acid levels during late infection stages may generate favorable conditions for biofilm formation. Indeed, the authors hypothesized that the low concentrations of the bile salt encountered during the natural restoration of microbiome may allow the cells to

transition toward biofilm formation. This would protect *C. difficile* vegetative cells against concentrations of antibacterial compounds that may normally inhibit growth (*i.e.*, antibiotics or other bile acids).[52]

In addition to promoting germination and inducing biofilm formation, bile acids can alter *C. difficile* physiology in other ways. Low amounts of LCA (0.08 mM) resulted in more elongated vegetative cells and absence of flagella. Fewer flagella were also observed when vegetative cells were incubated in 0.8 mM DCA and 0.3 mM CDCA. The authors hypothesized that lack of flagella may allow for better adherence to intestinal lining during infection. Additionally, bile acids resulted in up-regulation of the chaperon proteins DnaK, DnaJ, GrpE, GroL, and GroS, but the mechanism of up-regulation for chaperones is still unknown.[53]

Finally, bile acids inhibit TcdB toxin activity by binding to the toxin. Both conjugated and deconjugated secondary bile acids (e.g., DCA, LCA, GDCA, TDCA, GLCA, and TLCA) have greater potency in inhibiting toxin activity than their primary bile acid counterparts, but toxin binding by bile acids is reversible. The reversible binding and inhibition of bile acids to *C. difficile* toxins may thus suggest modulation mechanisms taking place in the gut depending on bile acid composition in the host.[54]

The role of bile acids in relation to *C. difficile* physiology and pathogenesis has been studied and characterized, extensively, since the discovery of their function as germinants for *C. difficile* spores.[16,46] Because of the many functions bile acids play in the host, it is not surprising that the interaction with *C. difficile* is complex and multifaceted, with both positive and negative interactions observed – depending on the conformation of, the abundance of, and the location of specific bile acids as well as the *C. difficile* life cycle stage in the host. More work should be done that move the field from data that correlate what bile acids are present in the host to potential causative interactions between bile acids and the host/*C. difficile*.

Stickland metabolites, amino acids, and the Wood Ljundahl pathway in *C. difficile* pathogenesis

Stickland metabolism was first identified in 1934 as the predominant pathway for *Clostridium sporogenes* energy production.[55] Stickland metabolism couples pairs of amino acids that may act as electron donors or acceptors.[56,57] The donor amino acid is oxidatively deaminated or decarboxylated to produce NADH, whereas the electron accepting amino acid is reduced to regenerate NAD^+ (Figure 2(a)). Although a myriad of amino acids can be used in the oxidative branch, the reductive branch is fueled only by proline or glycine. In the reductive branch, proline is consumed by proline reductase (PrdB, PrdA), a selenium-containing enzyme (selenoenzyme) that generates 5-aminovalerate and NAD^+, and glycine is consumed by glycine reductase (GrdA, GrdB), selenoproteins that generate acetate and NAD^+ (Figure 2(b, c)).[58,59]

Although Stickland metabolism is well-characterized in *Clostridium sticklandii*,[60] this alternative metabolic pathway in other Clostridial species has just recently been elucidated. Bouillaut et al.[59] analyzed the *C. difficile* glycine and proline reduction operons and their activity in the reductive pathway of Stickland metabolism. A mutation in the *prdB* subunit of the proline reductase enzyme resulted in a decrease in growth in rich media, but a mutation in a *grdA* mutant did not affect growth in rich medium.[59] Additionally, the authors characterize the sigma-54 dependent activator, PrdR that acts as a mediator for PrdB-dependent activation and proline-dependent toxin repression.[59] In addition, given the role the Stickland reductive branch has in regenerating NAD^+ for the cell, the redox dependent transcriptional repressor, Rex, has a role in proline-dependent regulation and is controlled by PrdR in *C. difficile*.[61] Using DNA binding assays and qRT-PCR, the authors found that proline seems to be the preferred amino acid for regeneration of NAD^+. In presence of excess proline, PrdR stimulates proline reductase expression and simultaneously Rex represses the glycine reductase gene *grdE*. NADH is then oxidized and, as a result, the ratio of NADH/NAD^+ is low. Alternatively, when levels of proline

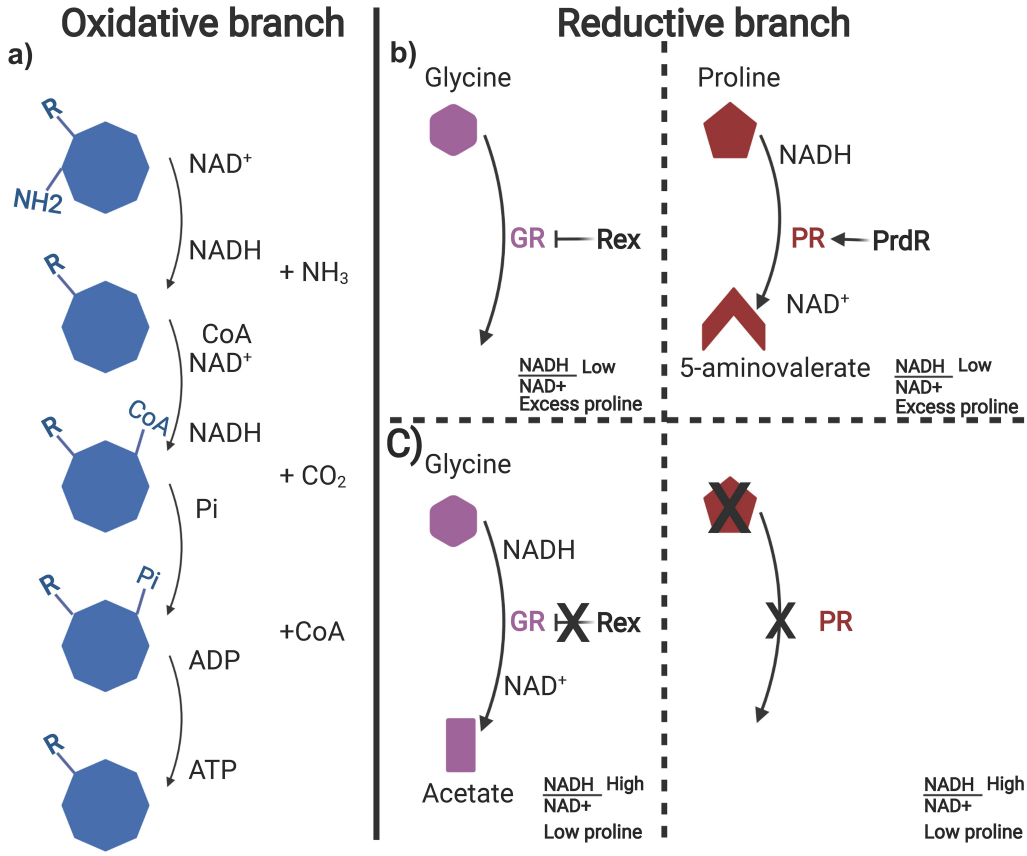


Figure 2. Oxidative and reductive branch of Stickland metabolism in *C. difficile*. Graphical representation of the oxidative and reductive Stickland pathways. A) Oxidation of a myriad of amino acids takes place in the oxidative branch of Stickland metabolism resulting in 2 NADH molecules and 1 ATP molecule produced. B) Proline and glycine are used to regenerate NAD^+ in the reductive branch. When excess proline is available in the surrounding environment and the NADH/NAD^+ ratio in the cell is low, the PrdR activator promotes proline reductase (PR) expression to generate 5-aminovalerate and NAD^+. Concurrently, Rex inhibits glycine reductase (GR). C) When proline levels are low and the NADH/NAD^+ ratio is high, Rex is unable to inhibit glycine reductase allowing acetate formation. Proline reductase activity is not present. Created with BioRender.com.

are low, the NADH/NAD^+ ratio increases and the high levels of NADH prevents Rex from repressing glycine reductase expression (Figure 2(b,c)).[61]

Selenium is an essential component of the proline and glycine reductases. Selenium is incorporated into these proteins as selenocysteine. Selenocysteine is generated through a synthesis pathway where the product of the *selD* gene reacts inorganic phosphate with hydrogen selenide to generate selenophosphate.[58,62,63] A *selD* mutant is unable to incorporate selenium into proteins and this results in a complete loss of the reductive branch of Stickland metabolism. The loss of selenophosphate generation results in a defect in the ability of *C. difficile* spores to outgrow following germination in peptide-rich media.[63,64] Also, the absence of selenophosphate altered *C. difficile* physiology so that other NAD^+ regeneration pathways were expressed.[64]

Several studies have correlated the depletion or increased levels of amino acids important for Stickland metabolism using both *in vitro* and *in vivo* approaches.[48,65–68] In an *in vitro*, multi-omics approach, the metabolome of multiple *C. difficile* growth stages found that Stickland metabolites were dramatically depleted upon entry into stationary phase[69] . Furthermore, in stool samples derived from hospitalized patients, amino acids used for Stickland metabolism were depleted in CDI patients, suggesting that these amino acids were consumed by *C. difficile* vegetative cells. In this study, branched chain amino acids, such as leucine, were hypothesized to be the preferred

amino acid used by *C. difficile*; other amino acids, such as proline, tyrosine, and phenylalanine are possible sources of energy for both *C. difficile* and other gut microbes.[67]

Proline availability is important for C. *difficile* colonization in mice. A mutation in proline reductase (*prdB*) resulted in decreased colonization in a humanized microbiome mouse model.[65] Moreover, *C. difficile* can take advantage of inflammation-induced collagen degradation. During this toxin-dependent process, the host cells respond to this inflammation by producing matrix metalloproteases. This results in the degradation of collagen – a protein rich in proline.[70]

Building upon this, in mice monoassociated with *C. scindens, C. hiranonis*, or *C. leptum*, proline was greatly depleted and mice were protected against CDI.[48] This work is also supported by *in vitro* conditions where *C. difficile* and *C. hiranonis* compete for nutrients.[71] Interestingly, Battagliogli et al.[65] observed high levels of the amino acids glycine, proline, threonine, and alanine in a dysbiotic colon, whereas Aguirre et. al.[48] observed depletion of proline and glycine in their monoassociated mice (amino acids essential for the reductive pathway of Stickland metabolism).[48,65] Nevertheless, direct comparison should not be made since one study used a humanized microbiome mouse model and the other used germ-free mice that were monoassociated with individual bacteria.

Expanding on the hypothesis that depletion of amino acids is important for Stickland metabolism, and may drive protection against CDI, Girinathan et al.[66] found that the gut commensal bacterium *P. bifermentans* protects against CDI, at least in part, through depleting nutrient sources used by *C. difficile*. Using *P. bifermentans* monoassociated mice, as well as carbon-source enrichment analysis of the gut-metabolomic environment, the authors found alterations in the substrates/products of Stickland metabolism (such as proline, glycine, threonine and 4-hydroxyproline) and production of 5-aminovalerate.[66]

Although *in vivo* FMT studies have reported levels of amino acids in FMT-recipients, such as increasing levels of valine, isoleucine, and leucine (amino acids able to be used in the oxidative branch), the presence of Stickland products in these recipients have not been reported [besides the SCFA (acetate, propionate)].[72–74] In a chemostat model, levels of 5-aminovalerate decreased post-FMT and isobutyrate levels remain constant post-FMT. A plausible explanation for the observed 5-aminovalerate levels could be the lack of Stickland fermenters included in the stable communities introduced into the chemostat, such as *P. bifermentans*.[75]

Expressed at lower levels when other forms of energy production are present, the Wood-Ljungdahl Pathway (WLP) may need examining during CDI. Through the WLP, two molecules of CO_2 are reduced to acetate.[76] The WLP can be coupled with butyrate production to allow for increased efficiency of ATP formation by decreasing nutritional requirements. Thus, the WLP may prove useful when glucose or amino acid levels are low, and *C. difficile* may need to adapt to low nutrient conditions.[77] Moreover, in a mouse model of CDI, the WLP increased expression twenty-four hours post-infection.[78] This suggests more attention should be given to this pathway in animal models, or in patient cohort samples.

Stickland metabolism by *C. difficile* during host colonization is well-established.[48,65,68] Nevertheless, the availability of the substrates needed for the oxidative and reductive branches of Stickland metabolism, and required compounds for WLP, during CDI likely shapes the ability of the bacterium to cause disease. Similarly, the presence of Stickland-using microbial competitors in the host microbiome also shapes how *C. difficile* colonizes a host.[48,66,79] Therefore, a better understanding of specific conditions in which *C. difficile* takes advantage of alternative metabolic pathways is necessary for the study of novel therapeutics that may modulate these pathways.

Non bile acid metabolites that correlate or show protection against *C. difficile* pathogenesis

The gut microbiome is complex, with interactions occurring between resident and invading bacteria that result in the generation of secondary metabolites. One such metabolite is coprostanol. Coprostanol is generated through the reduction of the double bond between C5 and C6 of cholesterol.[80] In the metabolome of healthy controls vs. CDI patients, 63 bacterial OTUs were

identified that positively correlated with the presence of coprostanol, which in turn negatively correlated with CDI patients.[81] The majority of phylotypes that correlated with coprostanol presence were members of the Lachnospiraceae and Ruminococcaceae families. Coprostanol may enhance resistance to CDI by decreasing the availability of cholesterol which could reduce the abundance of metabolites (*i.e.*, primary bile acids) that are necessary for germination by *C. difficile* spores (Figure 3(a,b)).[81]

Three microbial-derived SCFAs (propionate, acetate, and butyrate) have long been associated with CDI resistance.[82–85] In general, SCFAs are believed to play important roles in maintaining gut homeostasis.[86] Several studies have shown that SCFAs are depleted during CDI[5,81,86] Recently, butyrate, and acetate were found to protect against CDI by aiding in maintaining intestinal barrier integrity.[87,88] Butyrate plays a role in maintaining intestinal barrier integrity through limiting the permeability of intestinal epithelial cells to *C. difficile* toxins by stabilizing the hypoxia-inducible factor 1 alpha (HIF-1α).[87] Acetate acts during the early stages of *C. difficile* infection by activating the free fatty acid receptor 2 (FFAR2) signaling pathway by augmentation of IL-1B production from neutrophils and IL-1 R through signaling of ILC3. These two types of immune cells then induce production of IL-22 that is implicated in antimicrobial and repair mechanisms in intestinal epithelial cells.[88]

The mechanism by which propionate confers protection against CDI has yet to be elucidated. However, taking from data published on butyrate and acetate, a hypothesis could be that propionate acts upon specific host immune factors (Figure 3a). Interestingly, Gregory et al.[89] suggested that increasing levels of SFCAs as the gut microbiome recovers from disruption, may be used by *C. difficile* vegetative cells as a triggering signal to upregulate

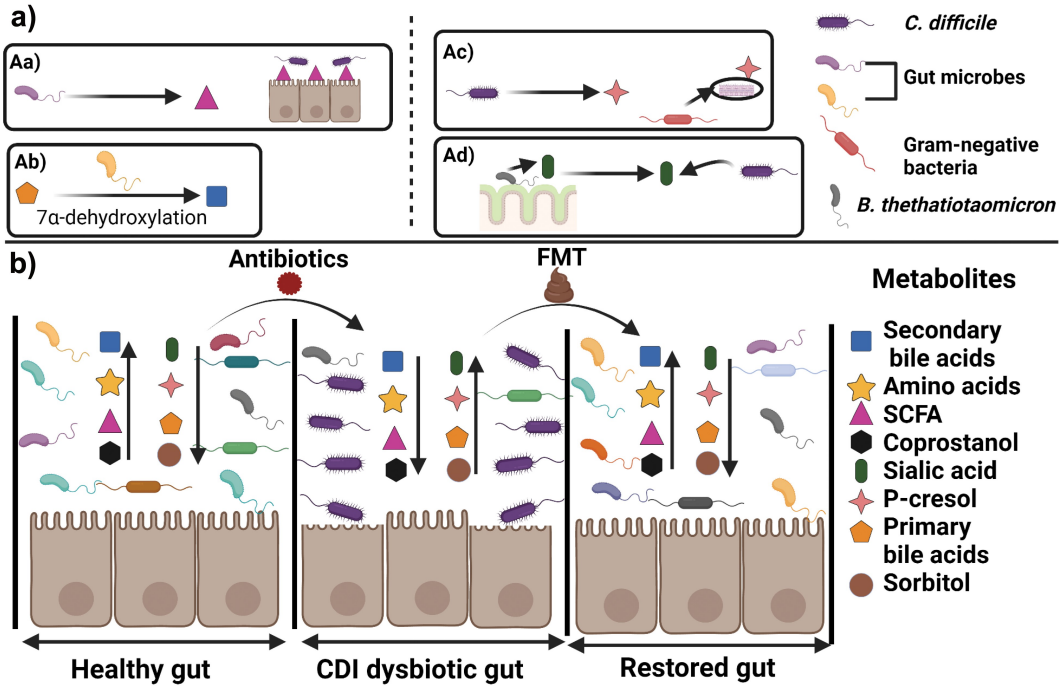


Figure 3. Gut metabolites effect in *C. difficile* growth and colonization. A) Examples of mechanisms of inhibition or protection against CDI. Aa) Gut microbes produce SCFA that aid in intestinal barrier function. Ab) Bacteria that perform 7α-dehydroxylation generate secondary bile acids that correlate with CDI resistant states. On the other hand Ac) generation of p-cresol by *C. difficile* may affect barrier function in Gram-negative bacteria, Ad) release of sialic acid from the colon mucus layer is used as a nutrient source by *C. difficile*. B) In an undisrupted gut environment amino acid, SCFA and secondary bile acid levels are high, creating a resistant state against *C. difficile* through immune defenses and nutrient limitation. High levels of the cholesterol derivative, coprostanol, are observed indicative of limited availability of primary bile acids. Introduction of antibiotics compromises the gut environment, and *C. difficile* toxins disrupt the intestinal barrier. *C. difficile* also promotes increasing levels of p-cresol, conjugated primary bile acids (*e.g.* TA) and sorbitol as well as use sialic acid as a nutrient source. FMT treatment allows restauration of gut metabolites and restore a resistant state against CDI. Created with BioRender.com.

toxin secretion and promote the inflammation that would allow the bacterium to maintain colonization in the gut.[89]

A recent study that used ^{1}H-NMR on stool samples derived from recurring CDI patients that received FMT, through capsule or colonoscopy, found increased levels of acetate, butyrate, and propionate in recipients 12-weeks post-FMT.[85] Interestingly, valerate, another SCFA was depleted in a chemostat C. *difficile* infection model.[75] Similar to the other SCFA, valerate levels were restored upon FMT. The study also found that valerate inhibited *in vitro C. difficile* vegetative growth in a dose-dependent manner, and that introducing valerate orally in the form of 15 mM glycerol trivalerate into a *C. difficile* mouse model decreased total viable counts.[75,85] It is important to note, though, that the glycerol trivalerate given to the mice was above physiologically relevant concentration since the authors found that chemostat FMT cultures only reached 4 mM valerate.

In addition to SCFAs, the microbiome produces antimicrobial compounds. Two Clostridial species, *C. scindens* and *C. sordellii* secrete tryptophan-derived antibiotic compounds (*i.e.*, 1-acetyl-β-carboline and turbomycin A) that inhibit *C. difficile* growth.[90] Interestingly, the anti-*C. difficile* effect of the antibiotics was enhanced by DCA and LCA. These findings suggest that a combination of secondary bile acids as well as antibiotics is at play during prevention of disease.[90] However, a recent study with mice that were monoassociated with *C. scindens* did not detect 1-acetyl-β-carboline in their metabolomics analysis,[48] suggesting that the activity of 1-acetyl-β-carboline may require specific conditions to be produced.

Dynamic interactions between gut microbes are constantly taking place and involve the production of and the sensing of secondary metabolites.[91] Although the mechanisms by which some of these metabolites protect against CDI are still unclear,[81,87,88,90] their identification provides important data that can be directly tested as potential treatment options for CDI. Moreover, a microbiome-centric approach for CDI treatment will most likely find other metabolites that show protection against CDI and could be explored for therapeutic approaches.

Metabolites and their beneficial roles for *C. difficile* infection

In recent years, the research focus on the modulation of the microbiome by *C. difficile* and how the bacterium survives in the host has increased.[92–96] *C. difficile* can produce products that promote inflammation and that have antibacterial effects. *C. difficile* generates *p*-cresol, a phenolic compound that affects the integrity of surface barriers in bacterial cells with a higher effect observed in Gram-negative bacteria.[93] The *hpdBCA* operon is responsible for the fermentation of tyrosine to *p*-hydroxyphenylacetate to then generate *p*-cresol using the 4-hydroxyphenylacetate dehydrogenase enzyme. In a mouse model of recurrent CDI, mice infected with a *hpdC* mutant strain, had increased microbial diversity compared to mice infected with the wildtype strain, as well as lower *C. difficile* viable counts. Additionally, when exogenous *p*-cresol was introduced to healthy human fecal slurries, the number of viable total anaerobes increased, thus suggesting that generation of *p*-cresol by *C. difficile* may give the bacterium a competitive advantage over other gut microbial species (Figure 3(a,c)).[93]

Similar to the production of *p*-cresol, production of sorbitol by *C. difficile* may enhance colonization. A *C. difficile* strain that is unable to produce sorbitol is outcompeted 10-fold by its wildtype counterpart in a mouse model.[94] Upon further investigation, the authors found that the inflammation induced by the *C. difficile* toxins results in an upregulation of aldose reductase which generates host-derived sorbitol. These results show how *C. difficile* is able to use a diet and host-derived nutrient to expand in the perturbed microbiome environment (Figure 3(b)).[94]

C. difficile also can use metabolites produced by the host and other gut microbes during colonization.[92,95,96] The gut symbiont *Bacteroides thetatiotaomicron* encodes a sialidase enzyme that cleaves and releases sialic acid from mucosal glycoconjugates. *C. difficile* encodes a sialic acid catabolic operon. Using a transcriptional analysis of germ free and *B. thetatiotaomicron* monoassociated mice, the data showed that the ability of *B. thetatiotaomicron* to release sialic acid resulted in increased expression levels of the *C. difficile* sialic acid operon.[95] Additionally, a spike in free sialic acid 1 day after

antibiotic treatment of mice was observed but these levels reduced 3 days after antibiotic treatment.[95] Moreover, mice monoassociated with a *B. thetatiotaomicron* sialidase mutant strain and then infected with *C. difficile* had lower *C. difficile* CFU counts (Figure 3(a-d)).[95] These results suggest an important role of sialic acid during *in vivo* infection.

Another host-produced metabolite, heme, can be used by *C. difficile*.[92] The heme-sensing membrane protein system, HsmRA, protects against redox damage generated from antibiotic treatment of CDI. *C. difficile* HsmA binds to heme that is released from the inflamed GI tract and shields the bacterium from redox-active molecules. Although the specific mechanism of protection by HsmA is still unknown, the authors hypothesized the use of HsmRA by other pathogens for protection against oxidative stress may be taking place.[92]

Finally, indole may play a role during *C. difficile* infections. Supernatant from *C. difficile* stationary phase cultures can induce the expression of tryptophanase (*tnaA*) in *E. coli*. The levels of indole increased in other indole-producing microbes in the gut (*e.g., L. reuteri* and *E. faecalis*) in co-culturing assays with *C. difficile*. Interestingly, the MIC (5 mM) of *C. difficile* strains were found to be higher than the MIC of multiple gastrointestinal bacteria tested, ranging from 2–4 mM. These results led the authors to the hypothesis that the ability of *C. difficile* to resist higher gut indole concentrations may provide the bacterium a competitive advantage.[96] Importantly, these experiments were performed under *in vitro* conditions and future work with animal models is needed to test the proposed hypothesis.

Successful *C. difficile* colonization, occurs when the host gut environment is disrupted. As a result, the bacterium must adapt and take advantage of resources available to maintain colonization and exclude the reestablishment of competing microbes.[97,98] The ability of the *C. difficile* bacterium to establish a niche in the gut environment by use of metabolites from intestinal epithelial cells, as well as secreted compounds to outcompete other gut microbes, suggests *C. difficile* is a bacterial generalist.[68] Although the factors that correlate with an environment that excludes *C. difficile* are well documented, the factors that *C. difficile* uses to exclude the microbiome from reestablishing itself are comparatively less understood. By inhibiting these mechanism, the normally-protective microbiome may gain a foothold and return the GI to a colonization resistant environment.

Conclusions and future directions

This review highlights specific metabolic compounds that have been regarded as important during the life cycle and pathogenesis pathway of CDI. As the research field, moves toward a microbiome-centric study of gut diseases, molecules used as nutritional sources for both pathogen and resident gut bacteria are being identified. In Figure 3, we show graphical representations of microbiome stages that may allow or prevent the gut environment to succumb to a *C. difficile* disease stage, as well as promote recovery.

C. difficile pathogenesis is complex. Although much work has focused on the effect that bile acids and antimicrobials have on the bacterium, considering the physiology of *C. difficile* for developing new treatments is emerging in the field. Potential treatment options against CDI have slowly progressed toward approaches that focus on restoring the gut microbiome ecosystem as a whole and not what affects *C. difficile*. Although some progress has been made, there are still many aspects to uncover regarding gut microbiota niche growth and competition in the GI tract. The move from broad-spectrum antibiotic treatment to more specific antibiotics, like ridinilazole that showed fewer CDI recurrences in a phase 2 trial, is happening at present.[99] Targeted molecules such as nanobodies or DARPins have also emerged and,[100] will probably become the norm for treatment against CDI in the future. Alternatively, specific strategies to reintroduce defined microbial communities to combat CDI, in the form of targeted microbiome therapies (e.g., SER-109, composed of purified Firmicutes bacterial spores),[101] might also work as an effective treatment strategy. Unraveling of additional bile salt biotransformations that can be taken advantage of to potentially modulate bile acid composition as well as bile acid analogs that do not undergo enterohepatic recirculation are yet other potential CDI treatments.[45,102] Finally, introduction of probiotics specifically targeting CDI or synbiotics (probiotics combined with prebiotics) that may modify the gut microbiome by increasing engraftment through introduction of specific nutrient sources appear to have potential for treatment in the future.[103,104] It is

important to mention though, that as understanding of the complex CDI pathogenesis and life cycle expands, the realization that individual treatments options may not be the way forward and comprehensive treatment plans may provide the best results, especially in regards to relapsing *C. difficile* episodes.

Disclosure statement

The authors report there are no competing interests to declare.

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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