1 Title

2 The emerging metabolic view of *Clostridium difficile* pathogenesis

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11 Abstract

12 It is widely accepted that *Clostridium difficile* exploits dysbiosis and leverages
13 inflammation to thrive in the gut environment, where it can asymptotically colonize
14 humans or cause a toxin-mediated disease ranging in severity from frequent watery
15 diarrhea to pseudomembranous colitis or toxic megacolon. Here, we synthesize recent
16 findings from the gut microbiota and enteric pathogenesis fields to inform the next steps
17 toward a better understanding of *C. difficile* infection (CDI). In this review, we present a
18 model in which the lifestyle of *C. difficile* is dictated by the metabolic state of the distal
19 gut ecosystem. Contributions by *C. difficile* (specifically the production and action of the
20 large glycosylating toxins TcdA and TcdB), the microbiota, and the host dictate whether
21 the gut environment is supportive to the pathogen. Mechanistic, metabolic pathway-
22 focused approaches encompassing the roles of all of these players are helping to elucidate
23 the molecular ecology of the distal gut underlying a diseased or healthy ecosystem. A

new generation of therapeutic strategies that are more targeted (and palatable) than fecal microbiota transplants or broad-spectrum antibiotics will be fueled by insight into the interspecies (host-microbe and microbe-microbe) interactions that differentiate healthy from pathogen-infested microbiotas.

The importance of understanding the metabolic ecology of *C. difficile*

The Centers for Disease Control and Prevention classifies *C. difficile* as an “urgent threat” to the nation’s health, as it causes over 450,000 infections yearly in the United States alone [1]. Dysbiosis, resulting most commonly from antibiotic use, is the primary risk factor for *C. difficile* infection (reviewed in [2]), highlighting the importance of the gut microbiota as a key mediator of CDI. However, up to 22% of CDI cases are not associated with recent exposure to antibiotics [3]; proton-pump inhibitors [4] and motility disturbance [5] have also been shown to induce dysbiosis leading to CDI. The increased appreciation for the role of the microbiota in CDI has informed the development and accelerated use of a number of novel therapeutics. Notably, fecal microbiota transplant (FMT) has proven to be remarkably effective in treating individuals with recurrent CDI and has received extensive recent coverage in scientific literature in the form of clinical trials [6,7] and in post-operative follow up studies tracking the microbiota of patients up to one year post FMT [8-12]. However, a lack of insight into the mechanisms of action of FMT, concerns over adverse events [13], lack of standardization, and cumbersome implementation suggest vast improvements in this therapeutic strategy are likely.

Importantly, recent studies have demonstrated that refined therapeutic strategies built upon the FMT concept successfully clear CDI to restore a healthy microbiota.

47 Outlined in the following sections, these examples support our model that *C. difficile*
48 metabolism within the context of the gut environment is integrally connected to
49 pathogenesis. In the first targeted restoration therapy, a specific cocktail of six
50 phylogenetically diverse fecal microbes ameliorated a murine model of persistent CDI
51 comparably to FMT; notably several other six-species cocktails failed to clear the
52 infection [14]. More recently, a single bacterial strain was shown to ameliorate murine
53 CDI in a secondary bile acid-dependent fashion [15]. In a study with a small number of
54 human patients, a 33-member bacterial community composed of strains isolated from a
55 healthy human donor effectively treated the infection [16]. However, despite these early
56 proof-of-concept studies showing that simplified bacteriotherapy can ameliorate CDI in
57 animal models and in humans, it is unclear how straightforward and generalizable a
58 defined microbial cocktail or single microbe will be across different patient groups.
59 Precision approaches tailored to different human populations (e.g., young vs. old or
60 immunocompromised) or across categories of CDI (e.g. acute vs. recurrent infection,
61 ranges of severity) may be required. An expansion in our understanding of the
62 mechanisms and associated ecology underlying the states that are supportive of and
63 inhibitory to *C. difficile* are likely to enable diverse new avenues to treat CDI, and will
64 more broadly inform our view of healthy versus diseased ecosystems. As we elaborate
65 below, it is possible that highly precise therapeutic approaches, such as those based
66 around metabolites or other small molecules, may be complemented by dietary strategies
67 that foster a healthy gut ecosystem to resolve or prevent CDI.

68

A hierarchical view of *C. difficile* pathogenesis: from community structure to toxin production

Signatures of different ecological states correlate with C. difficile status: 16S rRNA profiling

Given the importance of a healthy microbiota in resistance to CDI, descriptive studies aimed at understanding the microbial community structures underlying permissive and non-permissive states have provided an important foundation of knowledge. Based on their mechanisms of action, classes of antibiotics have distinct effects on the gut microbiota, making it possible to use antibiotics and the resulting differential effects on CDI susceptibility as a tool to identify specific bacterial taxa that render hosts susceptible or resistant to CDI. To identify taxa that distinguish susceptibility to CDI, Schubert et al. treated groups of mice, each with a different antibiotic to generate several distinct dysbiotic communities. These antibiotic-treated mice were subsequently subjected to experimental CDI, and patterns of community change associated with disease were identified. It was demonstrated that populations of *Escherichia* and *Streptococcus* were associated with CDI, whereas Porphyromonadaceae, Lachnospiraceae, *Lactobacillus*, and *Alistipes* were linked with protection [17]. A comparable strategy leveraging the differential effects of antibiotics on the microbiota was taken by Buffie et al., where members of the *Clostridium* cluster XIVa were strongly anti-correlated with antibiotic-mediated susceptibility to infection [15], supporting previous findings on the protective effects of members of this taxonomic cluster [18], which have been identified as important modulators of the immune system [19].

In another example [20], microbiome, clinical, and demographic data from 338 patients were integrated to reveal that the bacterial families Enterococcaceae, Enterobacteriaceae, Erysipelotrichaceae, and Lachnospiraceae are enriched in individuals with CDI relative to healthy controls and non-CDI diarrheal controls. Conversely, Bacteroidaceae, Lachnospiraceae, Porphyromonadaceae, and Ruminococcaceae were enriched in control groups. Notably, the Lachnospiraceae were associated with both CDI and healthy conditions, highlighting the need for delving beyond the high-level taxonomic resolution of 16S rRNA analysis to reveal underlying biology. What aspects of function, adaptation, or context distinguish related taxa that behave divergently during CDI? Furthermore, questions remain as to which microbes play a supportive role in CDI and which ones simply co-occur with *C. difficile*, possibly ‘hitchhiking’ on various aspects of the same environmental changes that fuel *C. difficile* growth. The pursuit of such questions will benefit from the use of additional highly controlled experiments in murine models of infection, which recapitulate many aspects of human disease and can be used in a gnotobiotic state for further experimental control.

Interrogating functional relationships with metabolomics

The mouse and human studies described above reveal that within studies, there are reproducible trends in the abundances of classes of microbes that differentiate CDI. These trends are imperfectly generalizable across studies, suggesting that additional microbial or host determinants are at play. Despite compositional differences, functional similarity of the gut microbiota, whether measured by metagenomic or metabolomic profiles, has been observed in healthy individuals [21,22]. A key question is whether

dysbiotic states susceptible to CDI are characterized by convergent changes in community functional capabilities. Indeed, despite large intra-group variability of microbiota composition among patients colonized with toxigenic or non-toxigenic *C. difficile* versus un-colonized diarrheal controls, each of the three groups possessed distinct but reproducible changes in community metabolism as measured by metabolite profile [23]. Of the identifiable metabolites, six chemicals were differentially abundant in *C. difficile*-colonized patients regardless of toxigenicity, relative to non-colonized controls: leonuriside A, *N*-palmitoyl glutamic acid, phlorizin, two tripeptides, and ceramide. On the other hand, patients colonized with toxigenic *C. difficile* harbored lower levels of choline and acetylputrescine, suggesting that *C. difficile* toxins engender a distinct metabolic ecological state. Further studies are necessary to validate the direct effects of *C. difficile* colonization and toxin production on changes in the abundances of the metabolites identified in this study, as well as their generalizability and how they fit into the microbial metabolic network during infection. Obtaining a detailed understanding of the distinct metabolic roles that *C. difficile* takes in the microbial community during different stages of infection will allow for therapeutic targeting using microbes or molecules that disrupt or enforce metabolic networks associated with disease and health (**Fig. 1**).

Mechanistic insights into inter-species interactions in gnotobiotic mouse models

Diverse metabolic interactions that occur in the gut can be either supportive or inhibitive of CDI. For example, *C. difficile* capitalizes upon a temporary availability of nutrients, such as the microbiota-liberated mucosal sugar sialic acid, that occur after

microbiota perturbation, to fuel its expansion [24]. *C. difficile* also takes advantage of an end product of commensal fermentation, succinate, to generate energy after microbiota disturbance caused by antibiotics or hastened gut motility [5]. Conversely, the commensal *Clostridium scindens* inhibits *C. difficile* growth by generating secondary bile acids toxic to *C. difficile* enhancing resistance to CDI [15]. Consistent with these findings, fecal microbial transplants (FMTs), to date the most effective treatment for recurrent CDI, have been shown to reconstitute a healthy fecal bile acid profile that is inhibitory to *C. difficile* spore germination and vegetative growth in CDI patients [25,26]. Because these metabolic interactions studied to date incompletely describe host susceptibility and resistance to CDI, they are likely a small subset of metabolites important in determining *C. difficile* fitness through different stages of infection and recovery (**Fig. 1**).

Pathogen-mediated inflammation catalyzes a switch in the metabolic landscape of the gut

Symptomatic CDI is primarily mediated by the action of two large glycosylating toxins, TcdAB, which inactivate the Rho family of GTPases. Many hypervirulent *C. difficile* isolates, particularly strains belonging to the epidemic 027 ribotype, also express binary toxin, an ADP ribosylating toxin recently shown to subvert the host innate immune response to *C. difficile* by indirectly inducing apoptosis in eosinophils [27]. As toxin-negative strains fail to cause disease and elicit a different metabolic profile than toxigenic strains [23], it appears that *C. difficile* utilizes toxin production as a strategy to alter the metabolic environment to support its growth.

Many enteric pathogens expand in the gut by first exploiting the dysbiotic intestinal food web (e.g. during antibiotic perturbation) to expand numerically and next by expressing virulence factors to create and maintain a modified niche that provides a competitive advantage over other community members [28]. In antibiotic treated mice, both *C. difficile* and *Salmonella enterica* serovar Typhimurium can grow to high levels in the distal gut during the first 24 hours of infection in the absence of virulence factor expression [29,30]. *S. Typhimurium* inflammation-independent post-antibiotic outgrowth was demonstrated using genetic mutants; similarly, the absence of toxin expression in wildtype *C. difficile* implies that toxin is regulated in response to cues in the gut, perhaps related to its own density and/or the density and metabolism of other microbes. After this early stage of CDI, the toxins are expressed at high levels. In support of the model that *C. difficile* toxin production is triggered by the need to sustain a metabolic environment favorable for its growth, there are many provocative regulatory links between environmental metabolites and TcdAB expression [31].

Case studies of *Salmonella* provide a foundation for future studies of *C. difficile* pathogenesis

Studies on *S. Typhimurium* have built a strong foundation for understanding inflammation-enabled metabolic strategies, providing a useful template for pursuing a more detailed understanding of *C. difficile* pathogenesis. Direct evidence for convergent metabolic strategies have been demonstrated for *C. difficile* and *S. Typhimurium*, with both bacteria capitalizing upon post-antibiotic availability of microbiota-liberated mucosal sugars during an intermediate state of infection ([24] and Fig. 1). During enteric

pathogen-mediated inflammation, the production of reactive oxygen and nitrogen species (ROS and RNS, respectively) associated with the innate immune response can react with compounds in the gut lumen to provide electron acceptors and enable aspects of anaerobic respiration not possible in the non-inflamed gut [32,33]. For example, ROS generated from *S. Typhimurium*-induced inflammation converts endogenous thiosulfate into tetrathionate, which the pathogen utilizes as an electron acceptor, bolstering its growth via anaerobic respiration [34]. Increased host production of RNS results in the generation of the oxidized sugars galactarate and glucarate, which also facilitate expansion of *S. Typhimurium* [35]. Ethanolamine, which is released from host tissue during inflammation, has been shown to confer a competitive advantage to and contribute to expression of virulence genes of both *S. Typhimurium* [36,37] and enterohemorrhagic *E. coli* O157:H7 [38], further suggesting that convergent metabolite-driven principles underlie strategies shared among multiple distinct enteric pathogens.

Not only does intestinal inflammation result in the production of novel nutrient sources readily metabolized by pathogens, but it results in reduced microbial diversity, presumably partly due to oxidative stress negatively impacting obligate anaerobes. Unlike the facultative anaerobes *S. Typhimurium* and *E. coli*, *C. difficile* is a strict anaerobe, suggesting that high levels of ROS in the lumen would be deleterious to this pathogen. It is possible that TcdAB-mediated inflammation and diarrhea similarly reduce the microbial diversity of the gut ecosystem by altering community composition to the advantage of *C. difficile*, however the underlying mechanisms are currently less clear and require further investigation. Strategies for ROS detoxification by *C. difficile* or other microbes during this inflammation, as well as the direct (privileged use of new carbon

sources or electron acceptors) vs. indirect effects (altered community composition enabling a competitive advantage for existing nutrients) of *C. difficile*-induced inflammation remain to be identified. It is likely that the inflammatory state induced by pathogens is tuned to favor each pathogen's fitness, matching multiple aspects of microbial physiology ranging from energy-producing metabolic pathways to oxidative stress tolerance.

Modulating the metabolic landscape of the intestine

Although, as described above, enteric pathogens alter the metabolic landscape of the gut through virulence, host diet represents a promising avenue to alter this environment in favor of a healthy intestinal ecosystem [39]. It was shown that dietary zinc plays a key role in determining CDI susceptibility and severity of disease [40] and that low protein diets are protective against CDI in mice [41]. Other studies highlighted the inhibitory effects of microbiota accessible carbohydrates (MACs) on *C. difficile* fitness by co-culturing *C. difficile* with *Bifidobacterium* spp. [42] or in the presence of a complex culture of fecal microbes [43]. MACs are present in the plant polysaccharides that make up dietary fiber and serve as the primary fuel for the gut microbial community [44]. In the absence of MACs, gut-resident microbes increase their consumption of intestinal mucus [45]. This metabolic switch is accompanied by significant changes in the membership/function of the gut microbiota and the loss of intestinal mucus leads to microbiota-dependent intestinal inflammation [46,47]. These observations illustrate the compositional plasticity and metabolic flexibility of this dense microbial population as modified by dietary input. Considering the utility of inflammation and metabolic

precursors in pathogen proliferation and persistence, it is reasonable to hypothesize that manipulation of MACs can modulate the fitness of enteric pathogens, including *C. difficile*. However, the extent to which MAC-centric interactions are relevant to *C. difficile* in the context of complex host-associated microbial ecosystems remains to be determined.

Synthesis and moving forward

Here, we propose a metabolism-centric model for *C. difficile* pathogenesis, whereby *C. difficile* produces toxin to catalyze a switch in the nutrient landscape to one that favors its own growth. Current data are consistent with this model in which expansion of *C. difficile* within the gut ecosystem is dependent on dysbiosis and that toxin production is a response to dynamics within a rapidly changing environment (Fig. 1). Under this model, *C. difficile* outgrowth occurs in the absence of toxin-mediated inflammation. After *C. difficile* successfully expands within the gut, virulent *C. difficile* may rely on toxin production to create inflammation to inhibit competitors or generate new nutrient sources. An interesting patient population that may give additional clues on the lifestyle of *C. difficile* in the absence of toxin-mediated disease are the healthy infants and adults that are asymptomatic carriers of toxigenic *C. difficile* (up to 90% and 15% of these populations, respectively) [48]. Further exploration into *C. difficile* and its interactions with the microbiota and the host in both diseased and non-diseased conditions is crucial to a better understanding of the transmission and pathogenesis of this organism. Elucidating molecular mechanisms in the context of the ecosystem dynamics

and nutrient landscape will help develop novel therapeutics that leverage microbes and molecules for improved management of *C. difficile*.

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Figure 1. A metabolism-centric model for the range of *C. difficile* lifestyles within the distal gut. Toxigenic *C. difficile* can persist in a range of environments in the distal gut. Future work to elucidate the mechanisms underlying these states will inform the development of novel interventions for the mitigation of CDI. **(A)** The microbiota of a healthy human may or may not contain toxigenic *C. difficile*. In asymptotically colonized individuals, the roles played by *C. difficile* in the intestinal ecosystem remain unclear. **(B)** The dysbiosis resulting from an exogenous disturbance (e.g., antibiotics) allows for *C. difficile* colonization and/or outgrowth in the distal gut in the absence of toxin production. At this stage, the gut ecosystem may return to a healthy stable state or *C. difficile* may express the large glycosylating toxins TcdA and TcdB. **(C)** *C. difficile* toxins lead to inflammation in the distal gut environment and are responsible for *C. difficile*-mediated disease. The mechanisms by which this inflammation favors *C. difficile* remain unclear but may involve direct (e.g. privileged use of oxidized carbon sources or electron acceptors) or indirect effects (e.g. altered community composition enabling a competitive advantage for existing nutrients). The goal of therapeutic intervention in the context of symptomatic CDI is to facilitate the path from a diseased to a healthy state. This may occur either through a transient intermediate dysbiotic state (e.g. antibiotic treatment) or by bypassing dysbiosis to stability (“smart” therapeutic targeting pathogen or its virulence factors, e.g. the selenoorganic compound Ebselen [49]). Prophylactic measures (e.g. dietary MAC consumption or targeted anti-virulence agents) may be taken to favor the healthy microbiota state in all individuals, especially those at risk for developing CDI.