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MINIREVIEW

From Nursery to Nursing Home: Emerging Concepts in *Clostridioides difficile* Pathogenesis

Alexander B. Smith,[a,b] Joshua Soto Ocana,[a,b] Joseph P. Zackular[a,b]

[a]Division of Protective Immunity, Children's Hospital of Philadelphia, Philadelphia, Pennsylvania, USA
[b]Department of Pathology and Laboratory Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, USA

Alexander B. Smith and Joshua Soto Ocana contributed equally. Co-first author order was determined alphabetically.

ABSTRACT *Clostridioides difficile* is a Gram-positive, spore-forming, anaerobic bacterium that infects the human gastrointestinal tract, causing a wide range of disorders that vary in severity from mild diarrhea to toxic megacolon and/or death. Over the past decade, incidence, severity, and costs associated with *C. difficile* infection (CDI) have increased dramatically in both the pediatric and adult populations. The factors driving this rapidly evolving epidemiology remain largely unknown but are likely due in part to previously unappreciated host, microbiota, and environmental factors. In this review, we will cover the risks and challenges of CDI in adult and pediatric populations and examine asymptomatic colonization in infants. We will also discuss the emerging role of diet, pharmaceutical drugs, and pathogen-microbiota interactions in *C. difficile* pathogenesis, as well as the impact of host-microbiota interactions in the manifestation of *C. difficile*-associated disease. Finally, we highlight new areas of research and novel strategies that may shed light on this complex infection and provide insights into the future of microbiota-based therapeutics for CDI.

KEYWORDS *Clostridium difficile*, microbiota, infectious disease

CLOSTRIDIOIDES DIFFICILE INFECTION

Clostridioides difficile (formerly known as *Clostridium difficile*) is a spore-forming anaerobic bacterium that infects the colon. In the United States, *C. difficile* is the most commonly reported nosocomial pathogen, and *C. difficile* infection (CDI) has emerged as an urgent public health threat worldwide (1). Over the past decade, the epidemiology of CDI has progressively evolved, and we are continuing to see increases in incidence, severity, and costs associated with infection (1, 2). Notably, non-antibiotic-associated CDI, most prominently in community-acquired cases, has been on the rise (2). In this review, we will discuss emerging concepts in *C. difficile* pathogenesis and epidemiology and provide insights into the potential factors contributing to the changes in epidemiology and rates of CDI.

C. DIFFICILE-ASSOCIATED DISEASE, RECURRENCE, AND CARRIAGE IN ADULTS AND THE ELDERLY

C. difficile-associated disease manifests as a wide spectrum of diseases that vary in severity from asymptomatic carriage to mild and moderate diarrhea to pseudomembranous colitis, toxic megacolon, and/or death (3). The primary risk factors for CDI are broad-spectrum antibiotic treatments, length of hospital stay, increasing age, and underlying comorbidities (4). CDI is most commonly reported in elderly hospitalized patients (3). Clindamycin, cephalosporins, ampicillin, and fluoroquinolones are the most highly associated classes of antibiotics with increased risk for CDI (5–7). *C. difficile*-associated diarrhea is the most common form of disease manifestation among patients (8). More severe presentations of CDI, such as pseudomembranous colitis, occur in a smaller subset of patients. Fulminant colitis, the most severe form of CDI-associated

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Address correspondence to Joseph P. Zackular, joseph.zackular@pennmedicine.upenn.edu.

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disease (9), occurs in approximately 3–8% of cases and accounts for the most serious complications of CDIs, including perforation of the epithelium, toxic megacolon, and death (10). Mortality rates for CDI have been on the rise over the past decade, which is correlated with the emergence of several epidemic strains of *C. difficile* (11, 12). These ribotype 027 (RT027) strains have been termed hypervirulent strains, but the link between these strains and increased disease severity has been questioned (13, 14). The emergence of RT027 is attributed to the acquisition of antibiotic resistance, specifically to fluoroquinolones (15). Within the adult population, the most susceptible group with the highest concurrent mortality rates is the elderly (11). This increase in risk is likely associated with decreased immune function, altered microbiota, and comorbidities.

One of the most substantial challenges in CDI is the high rate of recurrent infection. This is driven by the paradox that the primary risk factor for *C. difficile*, which is antibiotic treatment, is also the standard of care treatment for CDI. Approximately 25% of patients experience recurrent symptoms within 4 weeks after antibiotic therapy, and following each recurrence, rates of subsequent recurrent infection increase (1, 16, 17). In many cases, recurrence is attributed to infection with a different strain from the primary strain, suggesting continued microbiota perturbation following clearance, which allows for reinfection (18). However, most patients who relapse suffer from an infection with the same strain that caused the original episode (19, 20). Novel strategies for predicting and limiting recurrent infections are key for the future treatment of *C. difficile*.

Rates of colonization and asymptomatic carriage of *C. difficile* differ greatly between infants, adults, and the elderly population (21). In adults, carriage is quite rare and is associated with a myriad of risk factors, including prior antibiotic exposure, comorbidities, and age (21). Rates of carriage in nonhospitalized healthy adults are estimated to be between 0 and 15% of the population (22–27). These numbers creep to upwards of 0 to 50% when surveying the elderly population and cohorts in long-term health care facilities (17, 28–30). Extended hospital stays are also reported to be associated with increased rates of carriage, as continued exposure to spores and comorbidities likely increases the chance of transient colonization (27). It remains unclear if asymptomatic carriers of *C. difficile* are at a heightened risk for developing CDI following antibiotic exposure or if they represent a significant reservoir for *C. difficile* in the hospital or community settings.

C. DIFFICILE IN NEONATES AND INFANTS

A long-reported phenomenon in *C. difficile* epidemiology is the unusually high rates of asymptomatic colonization in neonates and infants. *C. difficile* was first isolated and characterized in 1935 from a healthy infant patient, emphasizing that this bacterium readily colonizes the infant gut (31). Studies vary in numbers, but it has been consistently reported that carriage rates in infants broadly range from 18 to 90% (32–36). This remarkably high rate of colonization is further convoluted by the fact that these infants harbor both toxigenic and nontoxigenic strains but rarely present with *C. difficile*-associated diarrhea. Surprisingly, neonates colonized with toxigenic strains of *C. difficile* harbor bacterial burdens at levels consistent with those in adults presenting pseudomembranous colitis (33, 37, 38). During the transition from neonate to infant, rates of *C. difficile* colonization decrease. Thirty-seven percent of infants younger than 1 month of age are colonized (33). Between 1 and 6 months of age, colonization decreases to 30%. During the period of 6 to 12 months, colonization decreases to 16%. After 2 years of age, the rates of asymptomatic colonization decrease to 10% (Fig. 1). The fact that the peak occurs during early life and decreases throughout development is indicative of ecological succession and maturation of the microbiota, as well as the maturation of host immunity.

Early in life, communication between the developing immune system and commensal organisms sets the stage for a symbiotic and beneficial relationship (39, 40). The microbiota early in development is highly dynamic in nature, and emerging evidence suggests that damage to this ecosystem early in life can promote acute and chronic

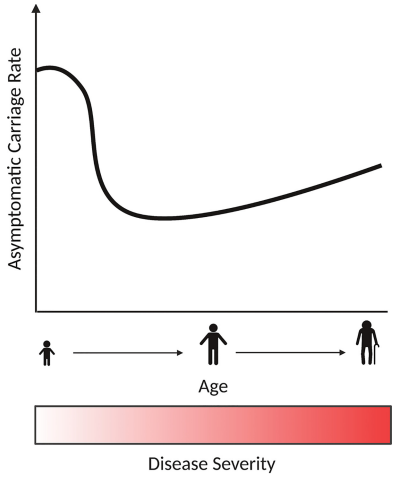

FIG 1 Relationship between asymptomatic colonization and disease severity of *C. difficile* with age. Higher rates of asymptomatic colonization are found in infants and neonates. As the microbiota and immune responses mature throughout development, changes in the bacterial community lead to a drop in colonization rates. In adults, colonization rates are low and increase in the elderly population. This is tightly associated with long-term stays in health facilities. Disease severity is also positively associated with age. While neonates and infants have higher carriage rates, they rarely suffer from infection. Meanwhile, adults and the elderly are the most susceptible population, and this is likely associated with alterations to the microbiota, decreased immune function, and comorbidities.

disorders later in life (41–44). Alterations in delivery method, introduction of antibiotics early in development, and shifts in nutrition have a marked impact on the community structure and homeostasis between host and microbiota (45–48). Due to the incredible complexity of the systems involved, we still know very little about the host, microbial, and nutritional factors that shape community assembly and pathogen colonization. It is likely that the early-life events in the microbiota play a key role in colonization and asymptomatic carriage of *C. difficile*, but more work is needed to shed light on the molecular mechanism associated with this phenomenon.

Although little is known regarding *C. difficile*-microbiota interactions at birth, the role of the microbiota in the transition from a carrier state to *C. difficile* clearance has been explored (36). In healthy babies, a reduction in *C. difficile* colonization at 1 year of age correlates with rising levels of *Bacteroides* and *Eubacterium* in the gut. Furthermore, *Bifidobacterium* and *Lactobacillus* are able to inhibit the growth of *C. difficile in vitro*, suggesting that early-life ecological events play a key role in reducing *C. difficile* colonization (49). It has also been suggested that breastfed babies may be more protected from *C. difficile* colonization than formula-fed babies, but the mechanisms by which breastfeeding prevents *C. difficile* colonization are unclear. Breastfed infants have a reduced mean fecal pH of 5.29 compared with pH 6.48 in bottle-fed infants, possibly related to the reduced buffering capacity of breast milk versus formula (50). Moreover, higher concentrations of IgA in breastfed infants can serve as a potent toxin A neutralizer (51, 52). In fact, a recent serological analysis of infants colonized with toxigenic strains of *C. difficile* revealed modest IgA and IgG response to toxins A and B, which could affect protection against infection later in life (53).

C. difficile carriage in neonates and infants represents a potential reservoir for *C. difficile* in both the hospital and community settings. Furthermore, asymptomatic colonization of infants with *C. difficile* could impact CDI risk, as carriers of toxigenic strains are at a higher risk for the development of infection than noncolonized patients (54). This is not clear-cut for infants, and data suggest even a potential benefit of asymptomatic colonization in infants, as antibody production can be protective against CDI (2, 55). Regardless, further research is needed to understand asymptomatic *C. difficile* colonization and if this acts protectively or represents a risk for developing CDI in infants.

FACTORS SHAPING *C. DIFFICILE* EPIDEMIOLOGY AND PATHOGENESIS

C. difficile disease falls on a wide spectrum of severity, and it is unclear what factors contribute to the severity of disease manifestation. Furthermore, the epidemiology of CDI has been rapidly changing over the past two decades, and the rates of both antibiotic-associated and non-antibiotic-associated CDIs are on the rise. Taken together, this suggests that previously unappreciated environmental factors may play a role in *C. difficile* infection and disease. In this section, we will highlight new evidence and emerging concepts that suggest a role for host, microbiota, and environmental factors in shaping CDI.

The microbiome and resistance to *C. difficile*. Following birth, the human gastrointestinal (GI) tract is rapidly colonized by a diverse collection of microorganisms with rich metabolic potential (39). This complex microbial community, termed the gut microbiota, aids in digestion, stimulates the immune system, and provides essential vitamins and nutrients to the host (56). The microbiota can play a key role in the metabolism and efficacy of pharmaceutical drugs (57), and perturbation to the homeostasis between host and microbiota is associated with metabolic disease, cancer, inflammatory bowel disease, and GI infection (58). Importantly, the gut microbiota also serves as an important ecological barrier to invading pathogenic organisms, such as *C. difficile* (59). Thus, the primary risk factor for CDI is antimicrobial use, which perturbs the microbiota and decreases resistance to *C. difficile*. During health, the microbiota provides protection against pathogens like *C. difficile* by facilitating the production of a variety of antimicrobial factors, stimulating the immune system, and directly outcompeting pathogenic bacteria for resources and niches (60). Following antimicrobial use, large shifts in the microbiota lead to ecological changes in the community and significant metabolic alterations. These perturbations decrease competition for nutrients, alter levels of *C. difficile* germination factors, and modify the immune response (61).

Microbiota-mediated resistance against *C. difficile* is tightly associated with the metabolic state of the GI tract. One of the most important classes of metabolites linked to CDI is bile acids, which represent a key gatekeeper for *C. difficile* colonization (62). Bile acids are cholesterol-derived, water-soluble molecules that are synthesized in the liver by hepatocytes and are secreted into the small intestine to aid in nutrient absorption for the host (63). Many host-produced primary bile acids, which are bile acids conjugated to glycine or taurine, are the major inducers of *C. difficile* spore germination (64–66). These primary bile acids are readily metabolized by members of the microbiota, which harbor a collection of enzymes for deconjugation and metabolism of secreted bile acids (62, 67). Secondary bile acids, which are the product of this metabolism, have been shown to directly inhibit germination and possess potent antimicrobial properties against vegetative *C. difficile* cells (68–71). Disruption of the gut microbiota alters the balance of these metabolic processes and can markedly change the ratio of primary and secondary bile acids, leading to *C. difficile* germination and initiation of disease (68, 70).

Emerging research has begun to circle in on specific members of the microbiota that play a central role in bile acid metabolism and resistance to *C. difficile* (68). The ultimate goal is to harness the metabolic potential of the microbiota to combat *C. difficile*. One such member is *Clostridium scindens*, a low-abundance member of the microbiota that is negatively correlated with *C. difficile* colonization (Fig. 2). *C. scindens* expresses enzymes in the secondary bile acid biosynthesis pathway and can confer resistance to *C. difficile* through remodeling of the metabolic pool in the GI tract (66, 68). *C. scindens* and related *Clostridia* represent only a fraction of the taxa in the microbiota that possess the capacity to manipulate the bile acid pool (67). Future studies focused on identifying members of the microbiota that can be used to restore resistance to *C. difficile* via bile acid metabolism have incredible promise in the treatment of CDI.

In addition to bile acids, several other microbial-derived metabolites have been demonstrated to play a key role in CDI. For example, recent studies have demonstrated

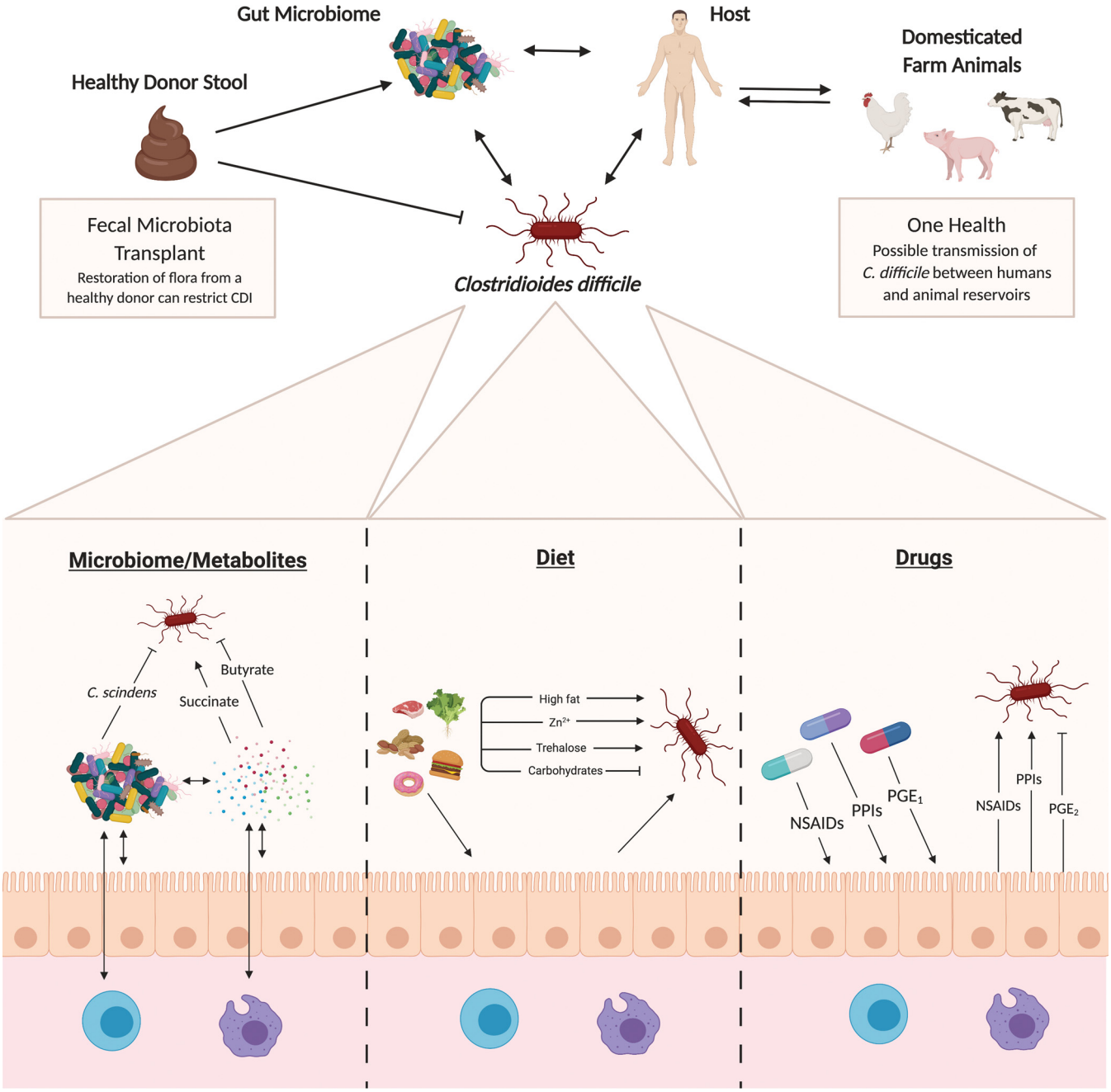

FIG 2 Factors influencing susceptibility to and severity of *C. difficile* infection. The factors that act to shape *C. difficile* susceptibility and infection severity through the tripartite relationship between the host, commensal microbiota, and pathogen are multifactorial. Bystander members of the gut microbiome, as well as their associated metabolites, are known to both positively and negatively modulate *C. difficile* pathogenesis. This is the basis for the function and activity of fecal microbiota transplant (FMT) as a therapy for *C. difficile* infection. Dietary factors, which can act directly on *C. difficile* or mediate interactions through the host, are also known to affect infection severity. Recent studies suggest that common drugs such as nonsteroidal anti-inflammatory drugs (NSAIDs) could impact disease progression. Lastly, emerging data suggest that bidirectional transmission of *C. difficile* strains between humans and animal reservoirs could play an understudied role in *C. difficile* epidemiology.

that antibiotic treatment leads to an enrichment in fermentable amino acids, such as proline, which are, in turn, utilized by *C. difficile* for energy in the GI tract (72). Additionally, it has been shown that upon antibiotic treatment, succinate levels are enriched in the GI tract of mice and associated with CDI (73). Specifically, it is postulated that perturbation to the microbiota leads to production of microbiota-derived succinate, which is utilized by *C. difficile* to enable expansion in the GI tract (Fig. 2). It has also been shown that sialic acid cleaved from the host mucus layer by members of the

microbiota can be cross-fed to *C. difficile* to enhance expansion in the gut (74). These interactions further exemplify how pathogen-microbiota metabolic interactions and the metabolic state of the gut can have a profound impact on CDI.

Polymicrobial synergy during CDI. In numerous infections, such as surgical wounds, otitis media, periodontal infections, and cystic fibrosis, interspecies interactions have been shown to drive physiological changes in bacteria that lead to the development of infection and induction of pathogenic states (75–78). This phenomenon is termed polymicrobial synergy, and these interactions can enhance pathogen persistence and exacerbate disease severity (78–80). Synergy between species can be driven by a number of mechanisms, including metabolite exchange, molecular signaling, or indirectly through the host. In periodontal infection, metabolite cross-feeding between the oral commensal *Streptococcus gordonii* and the opportunistic pathogen *Aggregatibacter actinomycetemcomitans* is critical for the establishment of infection and virulence (78).

During enteric infection, invading pathogens are exposed to a dynamic and rich polymicrobial environment. The bystander microbial community and polymicrobial interactions may play a key role in the outcome of CDI. As highlighted earlier, the microbiota plays an important role in antagonizing *C. difficile*, and our group has recently reviewed this topic in depth (126). However, our work and the work of others are beginning to explore how the antibiotic-perturbed microbiota may synergize with *C. difficile* to promote increased pathogenesis and negatively impact the outcome of infection. Specific taxa, including *Enterococcus* species and members of the *Enterobacteriaceae*, thrive in the *C. difficile*-infected gut and have been associated with susceptibility to infection (68, 81, 82). For example, following treatment with excess levels of zinc or different cocktails of antibiotics, *Enterococcus* becomes highly enriched in the microbiota of mice (68, 83). This enrichment of *Enterococcus* is correlated with increased susceptibility to infection and disease severity (68, 83). Despite these correlations, little work has been done to characterize the impact of bystander microbes on *C. difficile* pathogenesis, and the integration of microbiota-produced metabolic signals in disease outcome has not been thoroughly studied.

Immune response to *C. difficile*. Following colonization of the GI tract, vegetative *C. difficile* outgrows and produces its potent toxins, TcdA and TcdB (84). The pathogenicity locus (PaLoc) that carries the toxin genes also encodes a positive regulator, TcdR, and a negative regulator, TcdC. Moreover, toxin production can also be controlled through nutrient availability via global transcriptional repressors CodY and CcpA (84–86). Manifestation of *C. difficile*-associated disease is largely driven by a hyperinflammatory immune response to the damage caused by the toxins (87). These large multidomain toxins target Rho and Ras family small GTPases, leading to inactivation, subsequent actin disassembly, and eventual cell death (84). Toxin-induced damage to the epithelium creates a hyperinflammatory and volatile environment in the gut, loss of barrier function, and bacterial and toxin translocation (87). Depending on concentrations of toxin, cell death can be apoptotic or necrotic in intestinal epithelial cells. In macrophages, intoxication of epithelial cells and activation of the inflammasome lead to robust production of proinflammatory chemokines and cytokines, such as interleukin-8 (IL-8), IL-1β, and IL-22 (87, 88). This proinflammatory response leads to the robust recruitment of neutrophils during infection, a hallmark of CDI and pseudomembranous colitis, activation of innate lymphoid cells, and the production of antimicrobial peptides (87, 89, 90). The consequential innate immune response to CDI is essential for protection from translocating microbes, healing of the epithelial and mucosal barrier, and clearance of infection. However, this hyperinflammatory response is also a primary cause of much of the damage associated with CDI and can hinder recovery. Early response by innate lymphoid cells (ILCs), specifically ILC1s, is also critical in defense against *C. difficile* (91). The role of the adaptive immune response in CDI is less well understood, but studies suggest that the humoral immune responses to the *C. difficile* toxins may provide protection against disease and recurrence (92–94). Moreover,

neutralizing antibodies targeting TcdB, including the FDA-approved bezlotoxumab, have proven effective in treating CDI and blocking toxin activity (95–97). Despite this evidence, antibody responses do not seem to provide protection against colonization of *C. difficile* or alter the clinical course of CDI. Interestingly, clinical observations suggest that patients infected with *C. difficile* can develop disease that falls on a wide spectrum, ranging from asymptomatic colonization to severe colitis (3). It is hypothesized that differential immune responses to CDI likely play an important role in the outcome of infection. Understanding the factors that shape the immune response during CDI represents a major area of opportunity for the treatment of CDI.

Pharmaceutical drugs and CDI. Recent studies have begun to shed light on the unexpected effects that pharmaceutical drugs have on the gut microbiota (98). With susceptibility and severity of CDI being so tightly linked to the structure and composition of the gut microbiota, it is likely that pharmaceutical drugs play an unappreciated role in CDI (Fig. 2). One such example is nonsteroidal anti-inflammatory drugs (NSAIDs), which are among the most highly prescribed and most widely consumed drugs in the United States, particularly among older adults, and have been implicated in causing spontaneous colitis in humans (99, 100). Recent epidemiological studies have established an association between NSAIDs and CDI (101). NSAIDs act by inhibiting cyclooxygenase (COX) enzymatic activity, which prevents the generation of prostaglandins (PGs) and alters the outcome of subsequent inflammatory events. Prostaglandins, especially PGE_2, are important lipid mediators that are highly abundant at sites of inflammation and infection and that support gastrointestinal homeostasis. In the context of CDI, it has been shown that NSAIDs dramatically increase the mortality and intestinal pathology in mice. This is highlighted by alterations in the microbiota, prostaglandin dysregulation, altered proinflammatory profile, and decreased epithelial tight junction integrity (102, 103). Introduction of the stable PGE_1 analogue misoprostol protects mice from severe CDI and reduces microbiota perturbations (104).

Several retrospective analyses over the past two decades have found that patients prescribed proton pump inhibitors (PPIs) have an increased risk of contracting *C. difficile*, especially if they are on concurrent high-risk antibiotics (Fig. 2) (105, 106). PPIs increase the pH of the gastrointestinal tract by suppressing acid production. To date, no mechanism has been rigorously shown to explain any association between PPI use and increased incidence of CDI. It is becoming an increasingly controversial hypothesis, as other studies have found limited associations (107–109). The role of PPIs in CDI remains to be clarified, and follow-up studies are needed.

Evaluation of patients with depression who were on antidepressant medications demonstrated that utilization of certain antidepressant medications, such as mirtazapine and fluoxetine, is associated with CDI risk (110). This effect was independent of antibiotic exposure and particularly significant in patients taking both of these medications in combination. Taken together, these studies demonstrate the role of pharmaceutical drugs in risk and severity of CDI and highlight the potential role of these drugs in *C. difficile* epidemiology.

Diet and nutrition in CDI. Diet plays an essential role in shaping the microbiota, and interactions between the microbiota and dietary nutrients have been shown to be associated with numerous diseases (56, 111–114). Thus, it is not surprising that the impact of diet on CDI has become an emerging area of research in recent years. For example, a recent study demonstrated that a single micronutrient, zinc, given in excess to mice, dramatically alters the microbiota, increases susceptibility to CDI, and exacerbates disease (83). It is postulated that excess Zn alters the ecology of the microbial community, permitting *C. difficile* and other pathogenic microbes to thrive and cause severe *C. difficile*-associated disease (115). In an additional study, it was demonstrated that microbiota-accessible carbohydrates suppress *C. difficile* in the gastrointestinal tract by enriching for taxa that antagonize *C. difficile*. The availability of these carbohydrates leads to the production of metabolic end products, such as acetate, butyrate, and propionate, that decrease *C. difficile* fitness in the GI tract (116). Because of *C.*

difficile's ability to ferment amino acids as a nutrient source, dietary protein may also play a role in *C. difficile* pathogenesis. A recent study found that a high-fat/high-protein diet intensified *C. difficile* proliferation and virulence in a mouse model of infection (117). This study also supported the protective role of a high-carbohydrate diet. Finally, recent studies demonstrated that the dietary additive trehalose is an important mediator of the emergence of epidemic ribotype 027 (RT027) as well as ribotype 078 (RT078) strains of *C. difficile* (118, 119). Specifically, point mutations acquired by these highly virulent epidemic strains of *C. difficile* increased sensitivity to trehalose, allowing for utilization of this resource at low levels and selecting for emergence. Taken together, these studies have begun to shed light on the role diet can have on susceptibility to infection, the ecology of the *C. difficile*-infected gut, and pathogenesis and behavior of *C. difficile* in the GI tract.

***C. difficile* and One Health.** Epidemic RT027 strains of *C. difficile* have classically been associated with hospital-acquired infections, while an apparent role for RT078 in community-acquired infections is emerging (120, 121). Community-acquired *C. difficile* is on the rise, and though reservoirs such as asymptomatic carriers and animals are known, causal linkages have yet to be shown for the acquisition of *C. difficile* outside a hospital setting. However, the prevalence of *C. difficile* in domesticated animals, particularly agricultural animals, has sparked interest in how One Health (https://www.cdc.gov/onehealth/index.html) concepts can be applied to *C. difficile* (121). RT078, in particular, has a demonstrably high disease severity and attributable mortality at least as high as RT027 strains (122). Additionally, the identification of *C. difficile* RT078 strains and closely related lineages in the sequence type 11 group in domesticated animals such as pigs and cows adds a new level of complexity to the fight against antibiotic resistance (123). A recent study analyzing 247 *C. difficile* RT078 genomes from distinct geographic locations and hosts revealed a strong bidirectional correlation between human and animal strains with very little geographic clustering, indicating a high degree of intercontinental transmission (122). Data from a study in the Netherlands support this concept of interspecies transmission with the finding of clonal RT078 strains between pigs and farmers (124). Hospital-associated epidemics of certain *C. difficile* strains tend to emerge when they acquire resistance to high-risk antibiotics such as clindamycin and fluoroquinolones. However, with respect to RT078, there is an emergence of tetracycline resistance due to the heavy use of this antibiotic in agriculture (123). Another study, which sampled 400 RT078 genomes from across North America, Europe, and the United Kingdom, found that tetracycline resistance due to the *tetM* gene was by far the most abundant antimicrobial resistance marker among all the isolates, with resistance rates as high as 77.5% (123). These data highlight the emergence of antibiotic resistance in pathogens of human interest outside a hospital environment.

PERSPECTIVES

In this review, we highlighted recent evidence for the role of the pathogen, host, microbiota, and environment in the outcome of *C. difficile* infection and disease. Despite this growing evidence, defining the multidimensional interactions and molecular mechanisms in this complex ecosystem has proven to be incredibly challenging and has not moved far beyond simple associations. This gap in knowledge is particularly striking when one considers the broad and significant impact that *C. difficile* has on human health and health care systems worldwide. Furthermore, the continued rise of *C. difficile* incidence, high rates of recurrence, and emergence of hypervirulent strains highlight the need for identification of drug targets and the development of novel therapeutic strategies to treat this infection.

Several major focuses of the field, moving forward, center around further describing the role of bystander microbiota on *C. difficile* virulence and behavior during infection. Moreover, understanding the role of pharmaceutical drugs, diet, and nutritional status in susceptibility to and severity of CDI is paramount. A major theme that has emerged in *C. difficile* research and will continue to be a focus of our group and others is metabolic cross talk between the host, microbiota, and *C. difficile*. Metabolism and

metabolic interactions at the subcellular level form the basis for the function, survival, and behavior of all living cells (125). The role of metabolism and the importance of metabolic state are shared throughout the tree of life, and the building blocks for these behaviors are communal between immune cells, commensal bacteria, and invading pathogens. Thus, it is not surprising that metabolic cross talk between cells, species, and kingdoms has emerged as a key component of human health and disease. At no area in the body is this cross talk more evident than the GI tract. *C. difficile* interfaces with metabolites from the host, microbiota, environment, and diet, and each may directly impact this pathogen differently. Furthermore, metabolites produced by *C. difficile* and the microbiota can be sensed by the host, eliciting a myriad of cellular responses. It is well-known that microbiota-produced metabolites can even stimulate systemic responses in the host, including in distant tissues like the brain, but it is unclear how many of these metabolites may shape immunity to *C. difficile* during infection (58). Understanding how metabolites from the microbiota impact *C. difficile* and host immunity remains a major focus for the field moving forward.

In conclusion, it is clear that numerous variables impact susceptibility to this pathogen and the outcome of CDI. Future work will need to consider each facet of the tripartite interaction between the host, pathogen, and microbiota during infection. This will take continued development of novel methods that incorporate each of these variables, such as organoid models, intestine-on-a-chip systems, and advanced gnotobiotics. Furthermore, with the emergence of fecal microbiota transplantation as an incredibly successful treatment for CDI, it is clear that harnessing the ecological and metabolic potential of the microbiota will be at the forefront of therapeutic potential (Fig. 2).

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Alexander B. Smith earned a Bachelor of Science in biochemistry and molecular biology in 2018 from the Pennsylvania State University. While at Penn State, he studied the genetic determinants of Gram-negative bacterial membrane modifications in the laboratory of Dr. Timothy Meredith. Due to his interest in bacteriology and microbial ecology, he began his graduate training in the Microbiology, Virology, and Parasitology graduate group within the Cell and Molecular Biology program at the University of Pennsylvania. In 2019, Alexander joined the laboratory of Dr. Joseph Zackular. In the Zackular laboratory, Alexander studies synergistic microbial interactions between the enteric pathogen *Clostridioides difficile* and other members of the human gut microbiota.

Joshua Soto Ocana graduated from the University of Puerto Rico at Mayagüez in 2018 with a B.S. in Industrial Microbiology, a B.S. in Biology, and a minor in Biochemistry. During his undergraduate experience he worked on different areas of research which included investigating the role of insulin amyloid fibrils in type II diabetes, developing a green approach for the synthesis of indoles, and understating the role of IL-13 signaling in soft tissue sarcoma. Despite his undergraduate research experiences, Joshua knew he wanted to continue his graduate studies in microbiology. For that reason, he joined the Microbiology, Virology and Parasitology graduate group under the Cell and Molecular Biology Program at the University of Pennsylvania. In 2019, he joined Dr. Joseph Zackular's laboratory due to his interest in host-pathogen interactions. In Dr. Zackular's laboratory, Joshua is interested in understanding the role of pharmaceutical drugs on *Clostridioides difficile* infection, specifically looking at how nonsteroidal anti-inflammatory drugs can impact *C. difficile* pathogenesis.

Joseph P. Zackular, Ph.D., is an Assistant Professor in the Department of Pathology and Laboratory Medicine at the University of Pennsylvania and the Children's Hospital of Philadelphia. He received his Ph.D. from the University of Michigan where he studied the role of the gut microbiota in colorectal cancer in the laboratory of Dr. Patrick Schloss. He joined the laboratory of Dr. Eric Skaar at Vanderbilt University Medical Center for his postdoctoral fellowship where he studied the role of dietary metals and nutritional immunity in *Clostridium difficile* infection. In August of 2018, he started his laboratory in the Children's Hospital of Philadelphia. The Zackular laboratory is focused on understanding how interactions between the host, gut microbiota, and pathogenic microbes impact human health and disease. The lab's recent efforts center on understanding how the important nosocomial pathogen *C. difficile* interacts with resident gut microbiota during infection and how interspecies cross-talk impacts growth, behavior, and virulence of this pathogen. Research in the Zackular lab draws from a number of diverse fields including microbial ecology, bacterial pathogenesis, biochemistry, host-pathogen interactions, and microbiota research.