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Understanding the Roles of Nutrient-Niche Dynamics In *Clostridioides difficile* Colonization in Human Microbiome Colonized Minibioreactors

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UNDERSTANDING THE ROLES OF NUTRIENT-NICHE DYNAMICS IN *CLOSTRIDIOIDES DIFFICILE* COLONIZATION IN HUMAN MICROBIOME COLONIZED MINIBIOREACTORS

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Clostridioides difficile is a Gram-positive pathogen that is one of the most common causes of health care-associated infections in the U.S. Because of its importance to human health, researchers are trying to understand risk factors for infection and identify new therapies to treat disease. A "healthy" gut microbiome has the ability to protect the host against colonization of *C. difficile* or other pathogens by providing colonization resistance. One proposed mechanism for colonization resistance is through competition for nutrients with pathogens by the members of the healthy microbiome. We tested whether competition for nutrients was important for colonization resistance in human fecal minibioreactor arrays (MBRAs), an *in vitro* model of *C. difficile* colonization resistance that can be used to culture fecal communities from different healthy humans. We found that *C. difficile* growth was limited when amino acids were depleted and that ability to metabolize proline, an amino acid shown to be important in mouse models of infection, was required for *C. difficile* to colonize in some fecal communities but not others. As several previous studies point to proline as potentially important niche during human infections, these results provide further support for use of this model to understand factors important for *C. difficile* colonization that vary across fecal communities and can be used for further development of predictive models of important nutrient niches. Future

studies that build upon this work could combine sequence data, metabolomics data, and compound and pathway enrichment analysis to begin developing models that predict important nutrient niches in specific fecal communities as a way to develop personalized clinical therapy.

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1.1 Introduction

Clostridioides difficile is a Gram-positive, rod-shaped, obligately anaerobic bacterium that can produce toxins and form spores highly resistant to extreme conditions (Rupnik et al., 2009). Even though *C. difficile* has been characterized since 1935, it was not until the 1970s that there was evidence to indicate that C. *difficile* was pathogenic (Rodriguez et al., 2016). Ever since then till the end of the 20th century, the reported cases of *C. difficile* infection (CDI) increased slowly but steadily (Kelly & LaMont, 2008). In the early 2000s, rates of CDI began increasing significantly; of even more concern, was the rise of disease severity and morality rate (Kelly & LaMont, 2008). Because of these high rates of infection and severe disease, research has focused on understanding factors important for disease susceptibility to predict patients at risk for disease and to develop new therapies. In the rest of this review, I will highlight what is known about *C. difficile* infection, disease progression, and mechanisms for colonization resistance.

1.2 *Clostridioides difficile* Infection

1.2.1 History of *C. difficile*

In 1935, *C. difficile* was first isolated from the stool of a healthy newborn infant by Hall and O'Toole (Hall & O'Toole, 1935). Its original genus name, *Clostridium*, was given because it was a member of the anaerobic, spore-forming Gram-positive bacteria; its species name, *C. difficile*, was given to acknowledge that it is difficult to isolate and culture (Kelly & LaMont, 2008). Before the 1970s, there was no evidence to indicate that C. *difficile* was pathogenic and it was considered a member of normal human fecal microbiome (Rodriguez et al., 2016). In 1978, scientists investigating the cause of pseudomembranous colitis (PMC), a severe form of colitis in

which inflammation leads to pseudomembranes in the colon, established the association of *C. difficile* with PMC and demonstrated that previous antibiotic therapy is a key risk factor for susceptibility to infection (Bartlett et al., 1978; George et al., 1978). Ever since then till the end of 20th century, the reported cases of *C. difficile* infection (CDI) increased slowly but steadily (Kelly & LaMont, 2008). In the early 2000s, rates of CDI began increasing significantly; of even more concern, was the rise of disease severity and morality rate (Kelly & LaMont, 2008). This change in incidence, disease severity and mortality correlated with the emergence of new hypervirulent strains of *C. difficile* in North America and Europe that were classified as North American Pulsed Field Type 1 and PCR ribotype 027 (NAP-1/027) (McDonald et al., 2005). Several factors were hypothesized to contribute to the prevalence and severity of infection caused by this ribotype, including its ability to produce higher amounts of toxin, resistance to fluoroquinolones, production of binary toxin, hypersporulation and increased ecological fitness (Goudarzi et al., 2014; Kelly & LaMont, 2008; Robinson et al., 2014). While the prevalence of this ribotype has subsided to some extent, possibly due to reduction in usage of fluoroquinolones (Jassem et al., 2016; Wilcox et al., 2012), rates of *C. difficile* caused by multiple ribotypes remain high and are a significant concern for public health (Centers for Disease Control and Prevention (U.S.), 2019).

In the last decade, the increasing amount of sequence data from *C. difficile* and other sequenced *Clostridium* led to the recognition that the genus *Clostridium* was too broad and included organisms that were not closely related enough to be members of the same genus. In 2016, Lawson and coworkers proposed the re-classification of *"Clostridium" difficile* to *"Clostridioides" difficile* (Lawson et al., 2016; Oren & Rupnik, 2018). While there are still many authors that use the original name *Clostridium difficile*, *Clostridioides difficile* has been used

with more frequency over the past five years and is one of two recognized names for *C. difficile* (Oren & Rupnik, 2018).

1.2.2 Disease progression and pathogenesis

C. difficile is a Gram-positive, rod-shaped, obligately anaerobic bacterium that can produce toxins and form spores highly resistant to extreme conditions (Rupnik et al., 2009). *C. difficile* spores are often present in soils and other environments and its potential reservoirs include infected individuals, asymptomatic carriers, healthcare workers and contaminated environments (Czepiel et al., 2019). CDI patients usually acquire *C. difficile* spores through fecal-oral route (Seekatz & Young, 2014). Its spores pass through the upper digestive tract to the small intestine where they germinate into vegetative cells (Kochan et al., 2018). Under permissive conditions, vegetative cells can colonize the large intestine, produce toxins and cause diseases (Kochan et al., 2018). The infection can lead to various symptoms from mild diarrhea and abdominal pain to pseudomembranous colitis, toxic megacolon and even death in more complicated cases. While many infections resolve on their own, ~25% patients experience recurrent rounds of *C. difficile* infection (Johnson, 2009).

The major virulence factors produced by *C. difficile* are two exotoxins: ToxinA and ToxinB, which are encoded by *tcdA* and *tcdB* respectively, and are primarily responsible for the symptoms of CDI (Awad et al., 2014). These two genes are located in the pathogenicity locus as shown in Fig 1.1 (PaLoc), a 19.6 kb region of the chromosome that also contains accessory genes *tcdC*, *tcdR* and *tcdE* that encode proteins that regulate toxin production or toxin exportation (Awad et al., 2014; Braun et al., 1996). Both Toxin A and B belong to the large clostridial toxin (LCT) family which are monoglycosyltransferases (Carter et al., 2012). The

toxins act on target cells in the colon by glycosylation of Rho family GTPases, which results in an inactive conformation of the protein and therefore prevents the cellular activities that require the participation of Rho GTPases (Carter et al., 2012). Rho GTPases play important roles in many cellular processes, including the regulation of actin cytoskeleton assembly and organization (Pothoulakis, 2000). The intoxication of colonocytes causes disaggregation of filamentous actin and loss of structural integrity that results in death of intoxicated cells, which subsequently leads to epithelial cell disruption and impairment of cellular tight junctions (Carter et al., 2012; Pothoulakis, 2000). These effects together increase the intestinal permeability, which allows fluid accumulation that lead to diarrhea, a typical symptom of CDI in patients (Awad et al., 2014).

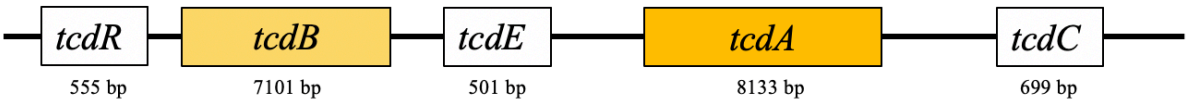

Figure 1.1. PaLoc region (19.6 kb). Genes in the pathogenicity locus (PaLoc) region of the *C. difficile* chromosome as described in the text.

In addition to the direct alteration of epithelium structure, toxin exposure also triggers the release of a number of chemokines and cytokines from epithelial cells and the migration of neutrophils to the site of infection (Voth & Ballard, 2005). The toxins could also activate mast cells and submucosal neurons following with the production of a variety of proinflammatory cytokines that lead to a profound inflammatory response (Awad et al., 2014). This response is an important characteristic of CDI and also correlated to the severity of host tissue damage (Awad et al., 2014).

Other factors that contribute to *C. difficile* disease severity include the Surface Layer Protein (SLP), flagella, and a third toxin called *C. difficile* transferase (CDT) that is produced by

some clinical *C. difficile* isolates including ribotype 027 strains. CDT ADP-ribosylates actin, which leads to loss of function and disruption of the actin cytoskeleton (Chandrasekaran & Lacy, 2017). Recent studies show that strains that produce CDT but do not produce TcdA or TcdB cause some intestinal hemorrhage, inflammation and fluid accumulation, but do not cause diarrhea (reviewed in Chandrasekaran & Lacy, 2017). Strains that only produce CDT and not TcdA or TcdB have also been found in patients with symptomatic *C. difficile* infection, but this is not very common. SLP and flagella both contribute to activation of the immune system (Solomon, 2013). Altogether, the toxins and other virulence factors disrupt the epithelial barrier and activate the immune system to varying extents across patients which leads to the variation in disease severity observed.

1.2.3 Epidemiology and risk factors of *C. difficile* infection

In the past two decades, the incidence and severity of CDI has considerably increased and this can be attributed to the spread of the hypervirulent ribotypes and excessive use of antibiotics (Goudarzi et al., 2014). CDI has now become one of the most common hospital-acquired infections (Centers for Disease Control and Prevention (U.S.), 2019). Identifying populations under higher risk for infection and severe disease will help with diagnosis and treatment with CDI patients. The most important risk factors for developing CDI include people older than 65 years old, male gender, antimicrobial therapy and prolonged duration of hospital stay (Goudarzi et al., 2014). In addition, populations with comorbidities or underlying conditions, such as immunodeficiency and HIV, inflammatory bowel diseases, neoplastic diseases, malnutrition, diabetes and cystic fibrosis are also considered high-risk populations for developing CDI (Lo Vecchio & Zacur, 2012). Among all these risk factors, the most common recognized risk factor is antimicrobial administration due to its disruption of indigenous gut microbiome; disruption of

the microbiome promotes *C. difficile* colonization (Goudarzi et al., 2014). A more complete description of how the gut microbiome typically resists *C. difficile* colonization is provided in more detail below.

1.3 Colonization resistance of commensal gastrointestinal microbiome

The gastrointestinal tract (GI) of humans is the harbor for trillions of microorganisms including bacteria, viruses, fungi and protozoa (Valdes et al., 2018). This collective community of microorganisms residing in the GI tract are referred to as the gut microbiota and this microbial community with their genomes are defined as gut microbiome (Valdes et al., 2018). After thousands of years of co-evolving, the microbiota developed an intricate, mutualistic relationship with its host (Bäckhed et al., 2005; Thursby & Juge, 2017). It has been estimated that the number of unique genes encoded by the gut microbiome can be over 100 times more than that of our own genome (Bäckhed et al., 2005). This large and diverse genomic content of gut microbiota allows it to provide various beneficial properties to the host, including assisting in maintaining the integrity of the mucosal barrier, development of the immune system, degradation of indigestible polysaccharides and providing colonization resistance (Thursby & Juge, 2017).

Colonization resistance is the term used to describe the ability of a healthy gut microbiome to provide protection against pathogens (Vollaard & Clasener, 1994). There are several potential mechanisms through which the gut microbiome can convey colonization resistance. The gut microbiome can mediate colonization resistance directly, by effectively competing for available nutrients and/or adhesion receptors on the intestinal epithelium, or via the production of growth-inhibitory or toxic substances, or the microbiome could mediate colonization resistance indirectly via the stimulation of host immune defenses (Stecher & Hardt, 2011). Studies in animal models and *in vitro* models have indicated that the indigenous

microbiome can inhibit *C. difficile* colonization (Borriello & Barclay, 1986; Wilson et al., 1981). Some specific mechanisms identified over the past several years are described below.

1.3.1 Potential mechanisms of colonization resistance

1.3.1.1 Inhibition of germination and growth through metabolism of bile salts by the gut microbiota

Since *C. difficile* spreads in the form of spores, which do not produce toxins that cause diseases, it is necessary for *C. difficile* to germinate into vegetative cells and outgrow in the GI tract before it can exert any influence on the host. The gut microbiome can inhibit this germination process by modifying bile acids that enter the large intestine (Sorg & Sonenshein, 2010).

Primary bile acids are synthesized in the liver, including cholate and chenodeoxycholate (Sorg & Sonenshein, 2010). To make them impermeable to cell membranes and therefore maintain a higher concentration of bile acids in the bile and gut, these primary bile acids are conjugated with either taurine or glycine, which are released to the digestive tract in response to food ingestion (Reed & Theriot, 2021). Although most bile acids will be absorbed for reutilization, a low concentration of bile acids still reaches the large intestine (Northfield & McColl, 1973). The gut microbiome plays two important roles in the transformation of bile acids. First, in the small intestine and proximal colon (cecum), the gut microbiome deconjugate bile acids by removing amino acids with bile salt hydrolase that is commonly encoded by gut bacteria (Ridlon et al., 2006). Secondly, a few species in the gut microbiome metabolize these deconjugated primary bile acids through 7α-dehydroxylation, converting cholate to deoxycholate and chenodeoxycholate to lithocholate (Ridlon et al., 2006). Therefore, the gut microbiome is

responsible for control the bile acids composition and ratio in the large intestine (Britton & Young, 2012).

Interestingly, these bile acids have different effects on germination process. Taurocholate, glycocholate, cholate and deoxycholate stimulate the spore germination while taurochenodeoxycholate, glycocenodeoxycholate, chenodeoxycholate and lithocholate show inhibitory effects (Sorg & Sonenshein, 2008). Though deoxycholate acts as a germinant, it is highly toxic to vegetative cells and cell death can occur quickly after germination in its presence (Britton & Young, 2012). Thus, only when taurocholate, glycocholate, and/or cholate levels in the environment are higher than other bile acids will *C. difficile* will be able to germinate and persist (Britton & Young, 2012). Through mediation of bile acids composition and ratio, gut microbiome provide protection by interfering with *C. difficile* spore germination and inhibiting vegetative growth.

1.3.1.2 Production of inhibitory metabolites

In addition to bile acid transformation, there are other commensal bacterial activities that produce metabolites that contribute to colonization resistance against *C. difficile*, such as production of short-chain fatty acids (SCFAs). SCFAs, mainly acetate, propionate and butyrate, are the main metabolites produced by commensal bacteria via the fermentation of indigestible polysaccharides like fibers and resistant starch (Silva et al., 2020). In a recent study in mice fed with a microbiota accessible carbohydrates-supplemented diet, the level of vegetative *C. difficile* cells in the feces was significantly lower along with an increased concentration of acetate, propionate and butyrate (Hryckowian et al., 2018). Further testing indicated SCFAs can directly inhibit the bacterial growth of *C. difficile* (Hryckowian et al., 2018). Although these three SCFAs increase expression of *tcdB in vitro*, the overall toxin level is reduced due to the decreased levels

of vegetative cells (Hryckowian et al., 2018). SCFAs also can lead to localized reduction of pH to lower than optimum pH for *C. difficile* growth, thus repressing the replication rate (Lawley & Walker, 2013). Additionally, there has been a recent study showing that butyrate can protect mice from *C. difficile*-induced colitis through reduction of intestinal permeability, inflammation and microbial translocation using an HIF-1 dependent mechanism (Fachi et al., 2019). This suggests gut microbiota produced SCFAs mediate colonization resistance not only via direct inhibitory effects, but also through regulating host intestinal structure and immune system function.

Some members of the gut microbiota also contribute to colonization resistance by producing antimicrobial metabolites that can inhibit *C. difficile* growth. One group of metabolites of particular interest is bacteriocins, which are peptides released by bacteria that have narrow or broad-spectrum bactericidal effect against pathogens like *C. difficile* (Lawley & Walker, 2013). Bacteriocins with a narrow-spectrum selectively target *C. difficile* and have potential to be novel antibiotics that can cure CDI without disrupting the indigenous microbiome and colonization resistance (Lawley & Walker, 2013).

1.3.2 Antibiotic disruption and impacts on the microbiome

Antibiotic administration plays a key role in CDI, not only is it the main risk factor related to clinical cases, but also antibiotic administration is required to make most experimental animal models susceptible to CDI (Theriot & Young, 2014). Several studies have shown that antibiotics have profound effects on the composition of gut microbiota. Mice treated with an antibiotic cocktail (ampicillin, gentamicin, metronidazole, neomycin, and vancomycin) via drinking water or oral gavage result in at least 10-fold reduction in total bacterial load in fecal samples (Hill et al., 2010). Sequencing results of this study suggest that antibiotic-treatment

introduced dramatic changes in terms of bacterial composition and diversity of gut microbiota, especially significant decreases in the levels of Firmicutes (Hill et al., 2010). In a separate study, fecal samples collected from human participants who received ciprofloxacin therapy also showed reduction in the level of Ruminococcaceae and Lachnospiraceae families that belong to Firmicutes (Dethlefsen & Relman, 2011).

The alteration of gut microbial community structure by antibiotics in turn results in changes of its metabolic functions, and therefore leads to changes in the metabolome. Mouse studies demonstrated that mice treated with streptomycin, gentamicin or ceftriaxone had drastic changes in metabolic profiles, including shifts in bile acids and amino acids metabolism, increases in oligosaccharides (sucrose, cellobiose, raffinose and stachyose), as well as decreases in monosaccharides (glucose, fructose, xylose and galactose) and SCFAs (Antunes et al., 2011; Zhao et al., 2013). It has been demonstrated that antibiotic treatment induces shifts in gut microbial metabolome and substances that potentially in favor of *C. difficile* sporulation and growth increase in abundance (Theriot et al., 2014). Taken together, antibiotics can alter gut microbial structure and metabolomic profile and therefore impair colonization resistance, thus increasing the susceptibility to *C. difficile* infection.

1.3.3 Competition and nutrient-niche theory

Another mechanism of colonization resistance is the competition for nutrients between the commensal microbiome and infecting *C. difficile* cells. This observation that colonic microflora suppresses *C. difficile* growth via competition for nutrients, including glucose, N-acetylglucosamine and sialic acids, was first highlighted in 1988 by Wilson et al. (Wilson & Perini, 1988). Since then, further studies have pointed to competition for nutrients as an effective mechanism to inhibit *C. difficile* growth. One good example is colonization with non-toxigenic

strains of *C. difficile*. In gnotobiotic mice, colonization of nontoxigenic *C. difficile* strain prior to challenge of toxigenic *C. difficile* strain successfully protected from disease. In human patients, oral administration of spores of nontoxigenic *C. difficile* significantly reduced the incidence of CDI recurrence in recovered CDI patients, suggesting that colonization of bacteria who share a similar nutritional requirement with *C. difficile* can effectively exclude *C. difficile* (Gerding et al., 2015; Wilson & Sheagren, 1983).

In addition to colonization with nontoxigenic *C. difficile*, colonization of *Clostridium bifermentans* before introduction of *C. difficile* also showed protective effect on gnotobiotic mice (Girinathan et al., 2020). The metabolomic results of mouse cecal content before *C. difficile* challenge illustrated significant decrease of nutrients in the *C. bifermentans* colonized group compared to the untreated control group. Of specific interest, amino acids (proline and glycine) that can be utilized by *C. difficile* via Stickland fermentation were largely depleted by *C. bifermentans* (Girinathan et al., 2020). Taken together, these studies provide evidence that supports the nutrient niche theory, which states that an organism must be able to utilize a subset of substances better than all competitors to colonize the intestine (Jenior et al., 2017). This means that a susceptible community must have one or more nutrient niches open up after administration of antibiotics that *C. difficile* is then able to fill in.

1.3.4 Amino acids as an open niche in disrupted commensal microbiome

C. difficile has been shown to use a coupled amino acid metabolism as its primary pathway to yield ATP (Jackson et al., 2006). This coupled amino acid metabolism is termed as Stickland fermentation (shown in Fig 1.2 (a)), where within a pair of amino acids, one amino acid is oxidatively deaminated as an electron donor while the other amino acid is reductively deaminated as an electron acceptor (Bouillaut et al., 2013). Theriot et al. found that increases in

glycine, proline, cysteine, isoleucine, valine, leucine and tryptophan, which are required for *C. difficile* germination and growth, is related to susceptibility to CDI (Theriot et al., 2014). In agreement with Theriot et al., another study showed that mice with dysbiotic gut microbiota are more susceptible to *C. difficile*, and this observation is correlated to the elevated concentrations of 12 amino acids, including alanine, proline, glycine, leucine and isoleucine, among which proline showing the greatest difference (Battaglioli et al., 2018).

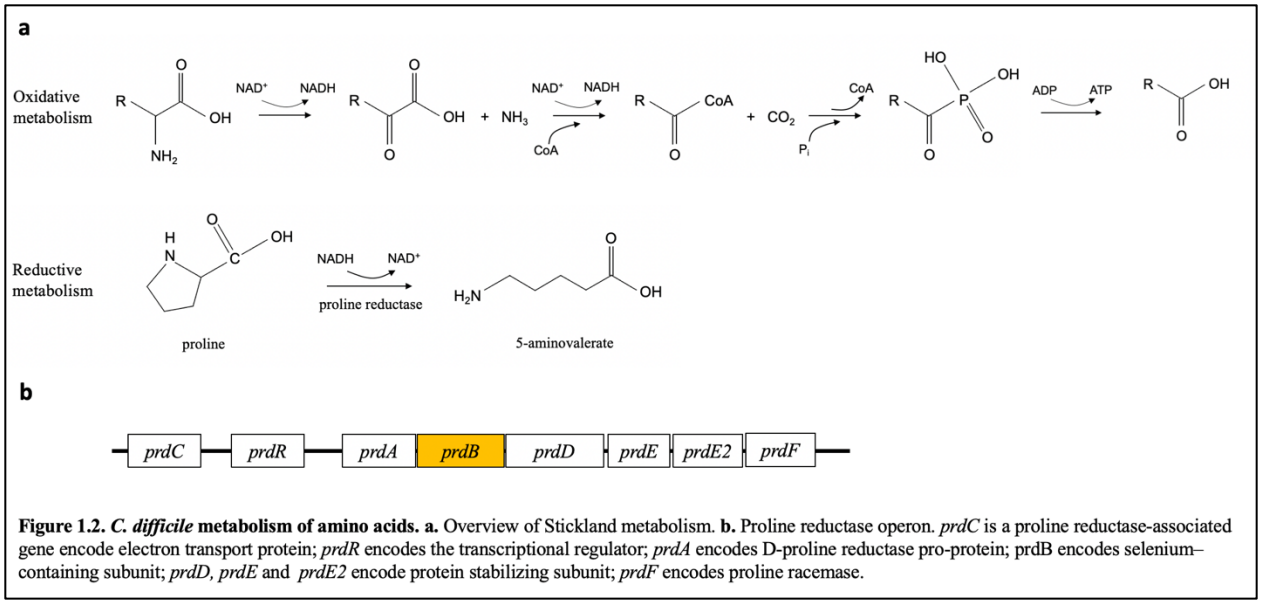

Figure 1.2. *C. difficile* metabolism of amino acids. a. Overview of Stickland metabolism. **b.** Proline reductase operon. *prdC* is a proline reductase-associated gene encode electron transport protein; *prdR* encodes the transcriptional regulator; *prdA* encodes D-proline reductase pro-protein; prdB encodes selenium–containing subunit; *prdD, prdE* and *prdE2* encode protein stabilizing subunit; *prdF* encodes proline racemase.

Proline is one of the most efficient Stickland acceptors; proline can be reduced by proline reductase (PR) (Jackson et al., 2006). In *C. difficile* the *prd* operon encoding PR consists of seven genes, details are shown in Fig 1.2 (b) (Bouillaut et al., 2013). *prdB* is one gene within the operon that is predicted to encode a subunit of PR (Bouillaut et al., 2013). In several mouse studies, *prdB* mutants of *C. difficile*, which are unable to use proline, showed decreased ability to colonize as well as a lower TcdB concentration compared to wild type *C. difficile*, indicating the ability of *C. difficile* to ferment proline is important to its colonization and virulence (Battaglioli et al., 2018; Lopez et al., 2019). Therefore, amino acids, especially proline, could be one of the

open nutrient niches important for *C. difficile* colonization in the intestinal environment after antibiotic treatment.

1.3.5 Modeling of human fecal microbiome using bioreactors to study *C. difficile*

1.3.5.1 Overall strengths and weaknesses of modeling microbiome *in vitro*

Since the late 1970s, *in vitro* models have been used by scientists to study *C. difficile* infection (Onderdonk et al., 1979). In the past decades, multiple systems have been developed and evolved into practical alternatives to animal models. Compared to animal models, *in vitro* models allow more precise control, higher throughput, easier operation and avoid the ethical issues about welfare of experimental animals (Best et al., 2012). However, the major shortcoming of *in vitro* models is that they cannot mimic interactions between host immune systems and gut microbiome during the process of disease development (Guzman-Rodriguez et al., 2018). In sections below, more details about several major types of *in vitro* models will be demonstrated.

1.3.5.2 Batch culture systems

Batch culture systems are the simplest *in vitro* system, but they also have the most limitations. Batch culture systems are closed system vessels with defined volumes; inoculation of feces into sterile media initiates the establishment of microbial communities (Guzman-Rodriguez et al., 2018). Since there is no replenishment of nutrients and waste is not removed, this kind of model usually are used in short-term studies analyzing microbial dynamics. Fecal emulsion is one of the batch culture systems; Borriello and Barclay used this model to study the importance of normal gut microbiome in preventing *C. difficile* colonization (Borriello & Barclay, 1986). They studied *C. difficile* growth in fecal emulsion derived from several groups of subjects, and found that fecal emulsion from healthy adults had higher inhibitory effects on *C. difficile* growth

than those from infants, children and geriatric patients while; this inhibition was removed by heating or filtration (Borriello & Barclay, 1986). Their study provided evidence that *in vitro* models can be used to examine the resistance of microbial communities to *C. difficile* infection and in studies investigating mechanisms of colonization resistance (Borriello & Barclay, 1986).

1.3.5.3 Single vessel continuous flow model

Compared to batch culture systems, continuous culture models are more physiologically relevant (Guzman-Rodriguez et al., 2018). Continuous culture models allow better controlled operation as well as prolonged experimental periods since fresh medium is replenished and waste is removed at a constant flow rate (Drake & Brogden, 2002). Thus, scientists are able to study organisms in an environment more reflective of conditions observed *in vivo* (Best et al., 2012). Freter and Wilson reported that introduction of hamster flora in germfree mice and continuous culture system colonized with *C. difficile* significantly repressed *C. difficile* growth (Wilson & Freter, 1986). They also showed that collection of isolates from hamster flora can repress *C. difficile* growth in both continuous culture model and germfree mice, suggesting that members of gut microbiome are responsible for colonization resistance (Wilson & Freter, 1986).

1.3.5.4 Three-stage continuous flow culture model

In the three-stage continuous flow model, there are three vessels that operate in a wier cascade system that is top-fed with growth medium at a controlled rate; each vessel is controlled at a specific pH to mimic the increasing alkalinity in the gut (Freeman et al., 2005). This system was validated against the cecal content of sudden death victims (Freeman et al., 2005). The three-stage continuous flow model allows the instillation of antibiotics at fecal or biliary levels that are reflective of a clinical dosing regimen, so this model has been used to study the ability of antibiotics to introduce CDI, and their efficacy as CDI treatments (Best et al., 2012). Wilcox and

colleagues used this model and hamster model to determine the efficacy of ramoplanin and vancomycin in clindamycin-induced *C. difficile* infection, and found that results from these two models are consistent (Freeman et al., 2005).

1.3.5.5 Minibioreactors arrays

Minibioreactor arrays (MBRAs) are a system of parallel single vessel continuous cultures that allow high throughput testing while maintaining complex microbial communities. It has been developed to operate at a small volume, thus it is easy to set up dozens of microbial communities simultaneously in an anerobic chamber to test different conditions with multiple replicates (Auchtung et al., 2015). Auchtung et al. demonstrated that MBRAs successfully cultured stable and reproducible microbial communities from different fecal donors (Auchtung et al., 2015). Using this model, Collins et al. found that dietary trehalose enhances virulence of epidemic *C. difficile* (Collins et al., 2018). Another study used this model demonstrated that administration of polyphenols reduces colonization resistance to *C. difficile* of the microbiome but can neutralize cytotoxicity (Mahnic et al., 2020)

1.4 Conclusion

C. difficile infections are still a significant problem to public health and research efforts are ongoing to understand disease susceptibility and potential treatments. One tool used to study risk factors and potential treatments are continuous-flow bioreactors inoculated with fecal samples from healthy humans. While these models have been used extensively as described above, relatively little is known about the mechanisms through which they prevent *C. difficile* colonization. Understanding these mechanisms are important for knowing how results obtained *in vitro* are likely to translate into human patients. In the next chapter, I will describe approaches that we used to show that nutrient niche dynamics can govern *C. difficile* colonization resistance

in vitro. Interestingly, I found that while metabolism of the amino acid proline is one important nutrient niche, this amino acid is not limiting in all fecal samples tested. Thus, the open nutrient niches for *C. difficile* in susceptible communities are probably distinct from one another across patients and further studies are needed to investigate these different nutrient niches and mechanisms for *C. difficile* colonization. Future studies based on this work could use analysis of sequence and metabolomics data from communities combined with compound and pathway enrichment analysis and the susceptibility of those communities to *C. difficile* to begin to develop predictive models for the important nutrient niches in a specific community and react accordingly to assist in preventing or treating CDI.

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Chapter 2 Investigating amino acids as a potential niche for *C. difficile* colonization in human microbiome colonized minibioreactors

2.1 Abstract

C. difficile is a Gram-positive pathogen that is one of the most common causes of health care-associated infections in the U.S. *C. difficile* infection (CDI) can lead to various symptoms from mild diarrhea and abdominal pain to pseudomembranous colitis, toxic megacolon and even death in more complicated cases. Because of its importance to human health, studies are trying to understand risk factors for infection and identify new therapies to treat disease. A "healthy" gut microbiome has the ability to protect the host against colonization of *C. difficile* or other pathogens by providing colonization resistance. One proposed mechanism for colonization resistance is through competition for nutrients with members of the healthy microbiome. We tested whether competition for nutrients was important for colonization resistance in human fecal minibioreactor arrays (MBRAs), an *in vitro* model of *C. difficile* colonization resistance that can be used to culture fecal communities from different healthy humans. We found that *C. difficile* growth was limited when amino acids were depleted and that proline, an amino acid shown to be important in mouse models of infection, was required for growth in some fecal communities and not others. As several previous studies point to proline metabolism as potentially important niche during human infections, these results provide further support for use of this model to understand factors important for *C. difficile* colonization that vary across fecal communities and can be used for further development of predictive models of important nutrient niches that can be modified to prevent or treat CDI.

2.2 Introduction

C. difficile is a Gram-positive, spore forming, toxin-producing anaerobic bacillus that can cause *C. difficile* infection (CDI) by acquisition of its spore through fecal-oral route (Seekatz & Young, 2014). The infection can lead to various symptoms from mild diarrhea and abdominal pain to pseudomembranous colitis, toxic megacolon and even death in more complicated cases. *C. difficile* was first recognized as causative agent of human disease in 1978 (Bartlett et al., 1978); now it has become the most common cause of health care-associated infections in the U.S. (Lessa et al., 2015).

Generally, a "healthy" gut microbiome has the ability to protect the host against colonization of *C. difficile* or other pathogens by providing colonization resistance. The gut microbiome can mediate colonization resistance through several potential mechanisms that were described in detail in Chapter 1. However, the exact mechanisms that enable the intestinal microbiome to limit *C. difficile* growth still remain less well understood and are actively being studied. A better understanding of the mechanisms of colonization resistance may provide insights to assist identifying at-risk individuals and boost their resistance by gut microbiota modification to prevent the spread of CDI.

The use of antibiotics can decrease or deplete some taxa of bacteria and alter the structure of the intestinal microbiome, which may directly lessen the competition for nutrients (Dethlefsen & Relman, 2011; Hill et al., 2010). In addition, it has been demonstrated that antibiotic treatment induces shifts in gut microbial metabolome and substances increase in abundance that potentially in favor of *C. difficile* sporulation and growth (Theriot et al., 2014). These antibiotic-induced changes of microbiome structure and metabolomic profile increase susceptibility to *C. difficile* colonization (Theriot et al., 2014). Unaltered microbial communities resistant to *C. difficile*

colonization provide evidence for the nutrient niches hypothesis states that an organism must be able to utilize a subset of substances better than all competitors to colonize the intestine (Jenior et al., 2017). This means that after administration of antibiotics, *C. difficile* is able to fill one or more nutrient niches opened up in a susceptible community.

C. difficile has been shown to use a coupled amino acid metabolism, known as Stickland fermentation, as its primary pathway to yield ATP (Jackson et al., 2006). One of the most efficient Stickland acceptors in *C. difficile* is proline, and proline concentration was shown to increase after antibiotic treatment in humans (Battaglioli et al., 2018). Further, *C. difficile* mutants unable to use proline due to inactivation of a subunit of proline reductase (Δ*prdB*) showed decreased ability to colonize in mouse studies compared to wild type, indicating the ability of *C. difficile* to ferment proline is important to its colonization (Battaglioli et al., 2018; Lopez et al., 2019).

Previous studies had shown that fecal samples cultured in continuous flow bioreactors from healthy humans can be used to model *C. difficile* colonization resistance and antibiotic-mediated susceptibility and to identify live microbes to treat *C. difficile* infection (Auchtung et al., 2020). While studies showed that competition for nutrient niches was important for colonization resistance in continuous-flow bioreactors colonized with mouse cecal contents (Wilson & Perini, 1988), it is not known if nutrient competition prevents *C. difficile* colonization across different human fecal communities cultured *in vitro,* or if other mechanisms, like production of bacteriocins or compounds toxic to *C. difficile* growth prevented colonization.

In earlier studies (Robinson et al., 2014), it was reported that *C. difficile* levels declined over time in human fecal minibioreactor arrays (MBRAs), suggesting that nutrient competition could be important for inhibiting *C. difficile* growth. We hypothesized that competition for

limiting nutrients inhibited *C. difficile* growth in human fecal communities. Because carbohydrates are limited in our human fecal MBRAs and amino acid metabolism is important for *C. difficile* growth *in vivo*, we also hypothesized that metabolism of amino acids, especially proline, could be one of the open nutrient niches important for *C. difficile* colonization in antibiotic-susceptible communities.

To test our hypothesis, we screened fecal samples from healthy humans to identify communities that exhibited resistance to *C. difficile* colonization in the absence of antibiotics, and susceptibility following disruption with the antibiotic clindamycin. We then used supplementation of spent culture medium from antibiotic-susceptible communities to determine that *C. difficile* growth was dependent upon supplementation of amino acids across all fecal samples tested. To precisely decide if proline metabolism was necessary for *C. difficile* colonization in MBRA communities, we compared levels of wild type, *ΔprdB* mutant (unable to ferment proline), and Δ*prdB* P_{tet}-*prdB* (*ΔprdB* mutant with restored ability to metabolize proline) over time in several MBRA communities. The results indicated that proline metabolism was required for colonization of some fecal communities but was not required to colonize other communities. We also tested whether introduction of *Clostridium sporogenes*, a bacterium that shares a similar nutrient requirement with *C. difficile*, prior to *C. difficile* challenge could interfere with *C. difficile* colonization. We found that *C. sporogenes* failed to prevent *C. difficile* colonization in the two susceptible communities tested. Taken together, our results suggest that amino acids could be an important niche for *C. difficile* colonization in some microbial communities while not in the others.

2.3 Materials and Methods

2.3.1 MBRA structure and operation

Each strip of MBRA consists of 6 separated chambers, each one with an influent port, an effluent port and a sample port. The total volume of each reactor is 25 ml and the working volume is 15 ml. As a continuous culture system, fresh media was replenished while waste was removed at a flow rate of 1.875 ml/h. To make sure the culture was well mixed, each reactor had a stir bar rotated continuously during the entire experimental period. Before use, the entire MBRA system along with bottled media was autoclaved to sterilize, then transferred into the anaerobic chamber at least 72 hours ahead of MBRA inoculation to achieve an anaerobic state. To inoculate, the reactors were filled with 15 ml fresh media, then 3 ml of freshly prepared fecal slurry was injected using syringe with needle. The preparation of fecal slurry is explained in detail below. After inoculation, initial community recovery and establishment was allowed for ~18 hours prior to the initiation of flow. The entire system was operated in heated anaerobic chamber under 37 °C with an atmosphere of 5% CO_2-5% H_2-90% N_2 (Robinson et al., 2014).

2.3.2 Fecal sample collection, preparation for MBRA experiments

Fecal samples donated by 7 healthy adults were collected with sterile containers, packed with GasPak (BD Biosciences) in sealed containers (8.1-quart; Sterile Ultraseal) on ice, and brought into the anaerobic chamber within 24 hours after collection (Robinson et al., 2014). Under anaerobic condition, samples were aliquoted into sterile cryovials (12 g/vial) and stored under -80 °C. Before MBRA inoculation, samples were brought into anaerobic chamber, suspended with anaerobic phosphate buffered saline (25% w/v). The suspension was vortexed for 5 min until homogenized, then centrifuged at ~200Xg for 10 minutes to remove large particles. The supernatant on top, referred to as the fecal slurry, was collected and used as

inoculum for MBRA. Fecal samples were collected from male and female subjects between the ages of 19-65 who were otherwise healthy and consented to participate in the study approved by the Institutional Review Board of the University of Nebraska-Lincoln (Protocol 18585).

2.3.3 Strains, media and growth conditions

For information of bacterial strains used in this study, see Table 2.1. **B**io**r**eactor **m**edium

Bacterial strain	Comments	Source
CD2015	027 ribotype, hypervirulent strain	Robinson et al., 2014
CD196	027 ribotype, hypervirulent strain	Lopez et al., 2020
CD196 *ΔprdB*	Mutant unable to ferment proline	Lopez et al., 2020
CD196 Δ*prdB* P_{tet}-*prdB*	Complementation of Δ*prdB*	This study
CD196 *rif*^{R}	Spontaneous rifampicin resistant mutant	This study
Clostridium sporogenes	Stickland fermenter	ATCC 3584
Clostridium butyricum	Non-Stickland fermenter, probiotic	ATCC 19398

Table 2.1 Bacterial strains

version **3** (BRM3) was used in all bioreactor experiments. Its complete and nutrient limited version was used in growth curve assays (Table 2.2). One liter of BRM3 contained two parts. The first part is 1 g tryptone, 2 g proteose peptone, 2 g yeast extract, 0.4 g sodium chloride, 0.5 g bovine bile, 1 ml 0.5% hemin, 1 ml 1% magnesium sulfate, 1 ml 1% calcium chloride, 2 ml Tween 80 dissolved in 950 ml water, which was autoclaved at 121°C for 60 min. The other part contains 47.8 ml water with 0.1 g arabinogalactan, 0.15 g D-cellobiose, 0.15 g maltose, 0.04 g D-glucose, 0.2 g inulin, 0.2 ml 0.5% vitamin K3, 2 g sodium bicarbonate, 1 ml 4% potassium phosphate dibasic, and 1 ml 4% potassium phosphate monobasic, which was filter sterilized and added to the first part following autoclaving (Auchtung et al., 2020)

Taurocholate-cefoxitin-cycloserine (TCCFA) agar was prepared as previously described (Robinson et al., 2014) and used to determine levels of *C. difficile* in MBRAs through serial dilution. TCCFA was supplemented with rifampicin (50 μg/ml) and erythromycin (20 μg/ml) for

plate counting CD2015, rifampicin (12.5 μg/ml) for counting spontaneous rifampicin resistant mutant of CD196 used in one set of competition experiments, and lincomycin (20 μg/ml) used to

Substance	BRM3	AA-limited	YE-limited BRM3	Sugar-limited
Tryptone	1 g	--	1 g	1 g
Proteose peptone	2 g	--	2 g	2 g
Yeast extract	2 g	2 g	--	2 g
NaCl	0.4 g	0.4 g	0.4 g	0.4 g
Bovine bile	0.5 g	0.5 g	0.5 g	0.5 g
0.5% hemin	1 ml	1 ml	1 ml	1 ml
1% $MgSO_4$	1 ml	1 ml	1 ml	1 ml
1% $CaCl_2$	1 ml	1 ml	1 ml	1 ml
Tween 80	2 ml	2 ml	2 ml	2 ml
Arabinogalactan	0.1 g	0.1 g	0.1 g	--
Cellobiose	0.15 g	0.15 g	0.15 g	--
Maltose	0.15 g	0.15 g	0.15 g	--
Glucose	0.04 g	0.04 g	0.04 g	--
Inulin	0.2 g	0.2 g	0.2 g	--
0.5% vitamin K3	0.2 ml	0.2 ml	0.2 ml	0.2 ml
$NaHCO_3$	2 g	2 g	2 g	2 g
4% K_2HPO_4	1 ml	1 ml	1 ml	1 ml
4% KH_2PO_4	1 ml	1 ml	1 ml	1 ml

Table 2.2 BRM3 recipe (1L)

detect *ΔprdB* mutants in competition experiments. *C. difficile* was routinely cultured in Brain Heart infusion agar and broth supplemented with 5% yeast extract (BHIS). For isolation of Δ*prdB* complemented strains (described below), thiamphenicol (15 μg/ml), cefoxitin (250 μg/ml), and cycloserine (16 μg/ml) were used to select for *C. difficile* transconjugants. LB agar and broth supplemented with appropriate antibiotics (20 μg/ml chloramphenical; 50 μg/ml ampicillin) was used for propagation of *E. coli* for cloning.

2.3.4 Construction of *prdB* complemented *ΔprdB* mutant

The *prdB* gene was amplified by PCR with NEBPhusion enzyme according to manufacturer's recommendation using chromosomal DNA of CD196 as template and primers 5'-ATATGAGCTCGAAAATAGAAGGGAGAGGAAATATATGAG-3' and 5'-ATATGGATCCCATTTTAAACGTGAGCTTTATATTCGTAT-3'. Amplifed DNA was digested with SacI-HF and BamHI-HF according to manufacturer's recommendations (NEB) and cloned into plasmid pRFP185 (Fagan & Fairweather, 2011) digested with the same enzymes. Plasmid and insert were ligated with T4 DNA ligase according to protocol (NEB) and transformed into NEB 5alpha high efficiency competent cells according to manufacturer's protocol. Plasmids were purified with Qiaspin miniprep kit according to protocol (Qiagen) and sequenced prior to transformation into competent *E. coli* SD46 cells, which contain the RP4 helper plasmid for conjugation as described in Collins et al. (Collins et al., 2018) Transformants were selected on LB ampicillin chloramphenicol and a single transformant was used for conjugation into CD196 Δ*prdB* strain as described in Collins et al. (Collins et al., 2018). The strain was grown on medium containing thiamphenicol except when preparing for use in MBRAs and for testing plasmid stability.

2.3.5 Determination of P*tet-prdB* plasmid stability

Before use of Δ*prdB* P_{tet}-prdB in MBRA experiments, we tested its plasmid stability to make sure it is stable enough so the *prdB* gene will be expressed and its ability to ferment proline is actually restored. The strain was inoculated in BRM3 broth with thiamphenicol (thi; 15 μg/ml) and let grow overnight. The overnight culture was diluted 1:20 in BRM3 broth with anhydrotetracycline (0.5 μg/ml; experimental group) and BRM3 broth with thiamphenicol and anhydrotetracycline (15 μg/ml and 0.5 μg/ml, respectively; control group) and incubated for 24

hrs. In the following two days, cells were diluted 1:20 every 24 hrs for two days for both groups. After the third diluted culture grew for 24 hrs, they were 10-fold serially diluted with phosphate buffered saline to 10^{-6}. For the diluent from 10^{-3} to 10^{-5} of both groups, 100 μl was spread plated on plain BHIS and BHIS with thiamphenicol. After overnight incubation, colonies on plates were counted. The plasmid stability is calculated with plasmid stability = (colony number of experimental group on BHIS Thi / colony number on BHIS) / (colony number of control group on BHIS Thi / colony number on BHIS). After three times of 1:20 dilution for every 24 hrs (12 generations), the calculation results of plasmid stability is 66.7%, which were determined stable enough for testing in MBRA.

2.3.6 Isolation of spontaneous rifampicin-resistant mutant

Since the wild type (wt) strain of *C. difficile* 196 is not resistant to the rifampicin concentration we normally use (50 μg/ml), we encountered problems when measuring *C. difficile* levels in competition experiments in MBRA (Because wild-type *C. difficile* levels were determined by subtracting the number of Δ*prdB* lincomycin-resistant colonies from the total population, we needed to use a lower estimation of wild type levels (1 colony out of total) for early days in experiment). Thus, we decided to select a spontaneous rifampicin-resistant mutant of wt CD196. By testing wt CD196 growth in BHIS with multiple concentrations of rifampicin, we found that the minimal inhibitory concentration (MIC) of rifampicin to wt CD196 is 2.5 μg/ml. Overnight wt CD196 culture was spread on TCCFA with 12.5 μg/ml rifampicin (5x of MIC), 100 μl culture per plate. Colonies formed after overnight incubation were considered as potential spontaneous rifampicin-resistant mutant. Those colonies were re-streaked onto TCCFA with 12.5 μg/ml rifampicin and let grow overnight. One colony was picked and inoculated into BHIS broth with 12.5 μg/ml rifampicin. Frozen stocks were made from the overnight BHIS

culture as well as cell pellet was collected and verified with PCR using *C. difficile* specific primers (5'-TTGAGCGATTTACTTCGGTAAAGA-3' and 5'-CCATCCTGTACTGGCTCACCT-3', amplify *C. difficile* 16S rRNA).

2.3.7 Spent medium collection from LBR

Since we needed a large volume of spent medium for testing and MBRA only contains 15ml in each reactor, a large bioreactor (LBR) model was used. Each fecal sample was inoculated into an LBR. The experiment followed the same experimental timeline shown in Fig. 2.2(a). On the first day, reactors were filled with fresh BRM3 to 150ml, then inoculated with 20 ml of of fecal slurry prepared as described above. After ~24hrs, the pump was turned on to start flow in reactors at a rate of 18.75 ml/hr. On day 3, clindamycin was added to source medium (250 ug/ml final concentration) and continued for 4 days, then switched back to fresh BRM3 for one day to wash out clindamycin. 100 ml of culture was removed from each LBR, centrifuged, and the supernatant was sterilized by filtration through a 0.2 μm PVDP filter under anaerobic conditions. The filtrate, referred to as spent medium, was sealed tightly and stored at -80°C until used for testing.

2.3.8 Growth assay using nutrient-limited medium

CD2015 growth in 100% fresh BRM3, 100% spent medium and 1:1 spent medium-fresh BRM3 mixture were used as control. CD2015 cultures were grown overnight in BRM3 medium. Overnight cultures were diluted 1:10 in control and test medium. CD2015 growth in 1:1 mixture of spent medium and three groups of nutrient limited-BRM3 (AA-limited BRM3, YE-limited BRM3 and Sugar-limited BRM3; details shown in Table 2.2) were obtained to see if there is a significant decrease of growth. Each condition had 8 replicates across two independent experiments. All growth curves were read with Tecan Sunrise plate reader at optical density of

600 nm (OD600) every 10 minutes for at least 24 hours. The maximum background-subtracted OD600 value was used as a measurement of growth yield.

2.3.9 *C. difficile* invasion in human microbiome colonized MBRA

Bottles of BRM3 were preincubated at 37 °C in the anaerobic chamber for at least 12 h to make sure there was no contamination. Before inoculation, the reactors were filled with 15 ml fresh medium and fecal slurry was prepared as described above. The inoculation was administrated with sterile needle and syringe, each reactor was injected with 3 ml of 25% w/v fecal slurry which resulted in fecal suspensions with a concentration at about 4% w/v. The freshly inoculated reactors were allowed to grow for ~18 h before the flow started, which gave the communities time to recover and establish. After ~36 h of the initiation of flow, clindamycin solution was added to those source bottles to reach a final concentration of 250 μg/ml and the treatment lasted for 3.5 days and stopped by switching source to fresh BRM3. After one day of termination of clindamycin treatment, *C. difficile* culture was introduced to each reactor. To prepare the *C. difficile* culture for invasion, BRM3 broth was inoculated with *C. difficile* and incubated at 37 °C overnight. 8 h before *C. difficile* challenge, a subculture was made by 1:10 dilution of the overnight culture. The subculture was 1:10 diluted again and used as culture for *C. difficile* challenge. Following challenge, *C. difficile* level was monitored on day 0, 1, 3, 5 by serial dilution and plating on TCCFA plates as indicated above. For competition assays, an equal volume of wt and Δ*prdB* mutant (based upon OD600) was prepared and inoculated into reactors. Day 0 values were obtained from the inoculum and used for determination of competitive indexes as indicated. In half of the competition experiments for FS515, FS685, and all replicates of FS235 and FS228 a spontaneous rifampicin resistant mutant of an otherwise wild type strain was used for competition. *C. difficile* levels were monitored on days 1, 3, and 5 by plating on

TCCFA and TCCFA + lincomycin (experiment with wild-type strain) or TCCFA + rifampicin and TCCFA + lincomycin (experiment with spontaneous rifampicin resistant mutant). For the competition experiment with wild-type CD196 strain, levels of wt were determined by subtracting the number of lincomycin-resistant Δ*prdB* colonies from total colonies, with a minimum ration of wild-type:Δ*prdB* colonies set at 0.05 when there were all detected colonies were lincomycin-resistant. For studies with Ptet-prdB, anhydrotetracycline was added to the medium beginning on day 0 at 0.25 ug/ml and each reactor was dosed with 300 ul BRM3 with 12.5 ug/ml anhydrotetracycline to keep it at a high enough concentration.

Fecal Sample	Wt	ΔprdB	ΔprdB/P_{tet}-*prdB*	Wt/ΔprdB competition
FS515	N=12	N=12	N=8	N=6
FS685	N=10	N=13	N=9	N=6
FS235	N=5	N=5	N=4	N=3
FS228	N=4	N=4	N=4	ND
FS133	N=3	N=3	ND	N=3
FS583	N=3	N=3	ND	N=3

Table 2.3. Replicates tested per fecal sample in each experiment

2.3.10 *C. sporogenes* and *C. butyricum* pre-colonization of MBRAs prior to *C. difficile* challenge

The experimental timeline is shown in detail in Figure 2.4(a). The pre-colonization of *C. sporogenes* and *C. butyricum* started one day after the termination of clindamycin treatment. Instead of introducing *C. difficile* at this time point as in other MBRAs experiments, here we added 300 µl overnight culture of *C. sporogenes* or *C. butyricum* to each reactor. In the following two days, overnight culture of *C. sporogenes* or *C. butyricum* were added every 24 hrs. After three times of dosing *C. sporogenes* or *C. butyricum*, MBRAs communities were challenged with wild type strain of *C. difficile* 196. The *C. difficile* levels were monitored till day

5 after *C. difficile* introduction. Levels of *C. sporogenes and C. butyricum* were measured prior to dosing into reactors and found to be 8.8 x 10^6 CFU/ml.

2.3.11 Statistical analysis

Statistical analysis was performed in GraphPad Prism version 9.0 as indicated in the figure legends. Unless otherwise indicated, a two-tailed, unpaired Student's t-test with Dunn's correction for unequal variance was used. P-values less than 0.05 are reported.

2.4 Results and Discussion

2.4.1 Identification of communities with *C. difficile* colonization resistance and susceptibility

Previous studies (Auchtung et al., 2020) showed that communities cultured *in vitro* from different healthy human fecal samples are either resistant to *C. difficile* colonization or susceptible to colonization without any antibiotics. It is important to identify communities that have indigenous colonization resistance when there is no antibiotic disruption to better understand the roles that specific nutrients may play in colonization resistance in MBRAs. Therefore, we examined colonization resistance of communities from seven recently donated fecal samples from healthy humans in MBRA. Communities were stabilized for one week and then inoculated with *C. difficile* as shown in Figure 2.1 (a). In four of seven tested fecal samples, levels of the ribotype 027 strain *C. difficile* 2015 (CD2015) decreased more than 1000-fold by day 5 in culture to an extent that was under our detection limit, indicating these four fecal communities have colonization resistance against *C. difficile* (Fig 2.1 (b)). As shown in more detail below, these four communities are susceptible to *C. difficile* infection following treatment with the antibiotic, clindamycin.

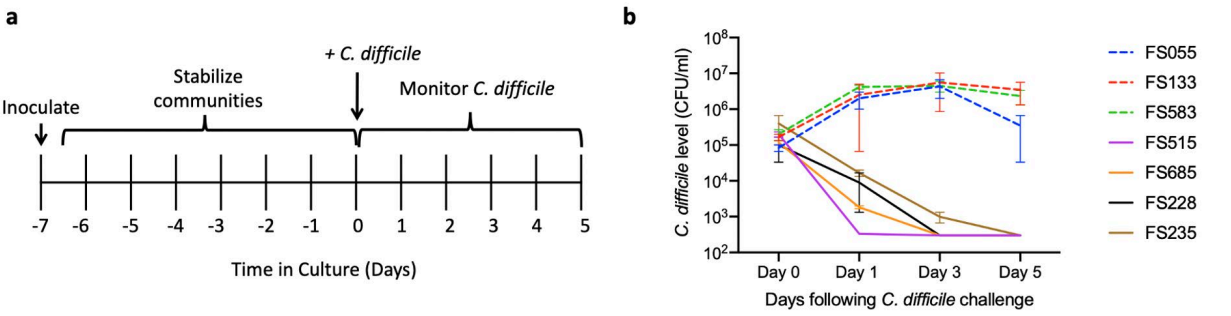

Figure 2.1 a. Overview of MBRA time course to examine response of communities to *C. difficile* colonization. **b**. Mean growth of CD2015 ± SEM over time in 7 fecal samples. N = 2 replicates for each fecal sample.

2.4.2 Nutrient depletion inhibits *C. difficile* growth in MBRA spent medium

In initial studies to determine if *C. difficile* growth is governed by nutrient-niche dynamics, we tested *C. difficile* growth in spent medium. For these studies, we used larger volume bioreactors that operate similar to MBRAs to collect large volumes of cell-free spent medium from fecal communities that were treated with the antibiotic clindamycin as outlined in Figure 2.2(a) and described in Methods. As shown in Figure 2.2 (b)-(d), spent medium itself could not support *C. difficile* growth. However, when cultured in 1:1 mixture of fresh medium and spent medium, *C. difficile* growth in 2 among all 3 tested fecal samples was not significantly different compared to growth in 100% fresh medium. (Growth in in the third fecal sample tested increased, but was not at the level observed in 100% fresh medium). This indicates that nutrients in spent medium were depleted to a level that was too low to support cell growth, but the addition of fresh medium could fully (FS228, Fig2.2(b) and FS615, Fig2.2(d)) or partially (FS515, Fig2.2(c)) restore growth. Then, we tested if there is a specific category of nutrient in

fresh medium that contributed to its ability to restore growth. We made three groups of nutrient limited bioreactor medium (amino acid-limited [AA limit], yeast extract-limited [YE limit], and carbohydrate-limited [CA limit]; details shown in Table 2.2) and tested *C. difficile* growth in 1:1

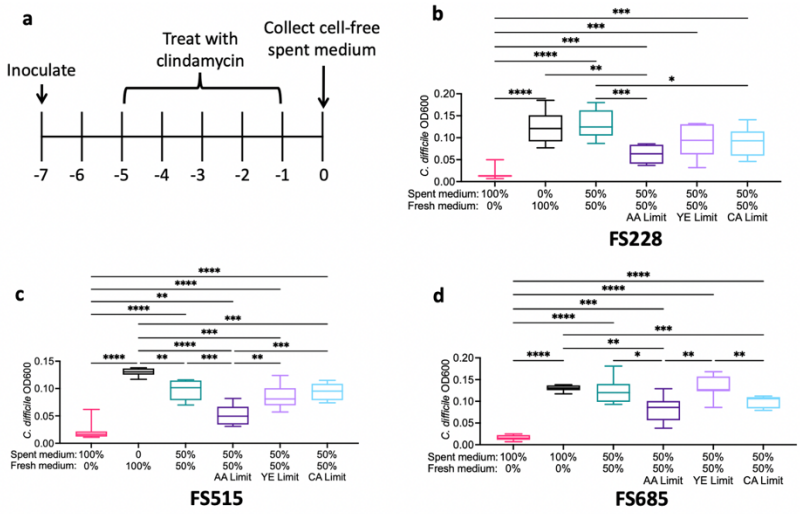

Figure 2.2 Nutrient depletion limits *C. difficile* growth in spent culture medium. **a**. Overview of large bioreactor time course prior to collection of spent culture medium. **b-d**. Analysis of maximum *C. difficile* growth (Optical Density at 600 nm; OD600) in spent medium collected from FS228 (**b**), FS515 (**c**), or FS685 (**d**). Growth comparisons were between cells grown in 100% spent medium, 100% fresh medium, or 50% spent medium + 50% fresh medium. For 50:50 mixing, fresh medium composition varied as indicated in Table 2.3 and was either complete, or lacked, amino acids (AA-limited), yeast extract (YE-limited), or carbohydrates (CA-limited). N = 8 replicates for each condition. Statistical significance was determined by Student's t-test with Welch's correction for unequal variances; *, p<0.05; **, p<0.01, ***, p<0.001; ****, p<0.0001.

mixture of spent medium and nutrient limited-BRM3. The results showed that growth decreased significantly when *C. difficile* was cultured in spent medium from all the three fecal samples supplemented with an equal volume of amino acid-limited medium compared to complete bioreactor medium. *C. difficile* growth was also significantly decreased when spent medium from FS228 was mixed with carbohydrate-limited medium compared to growth when spent medium was mixed with fresh medium. There were no significant differences in *C. difficile* growth when spent medium was mixed with YE-limited medium compared to growth when spent medium was mixed with fresh medium in any fecal community. These results suggest *C. difficile* was not able to grow as well mostly due to lack of amino acids, and that amino acid metabolism could be an

important nutrient that defines *C. difficile* colonization niche in clindamycin-treated human fecal microbiome colonized MBRAs.

2.4.3 Proline metabolism is a variable requirement for infection in MBRAs

Because we found that amino acids could be important nutrient niches for *C. difficile*, we investigated if metabolism of the amino acid proline could be important for *C. difficile* colonization. As described in detail in Chapter 1, recent studies have shown that proline is an important amino acid metabolized by *C. difficile* during mouse infections. In addition, a recent study tested the ability of wild type (wt) *C. difficile* strain 196 and its isogenic mutant unable to utilize proline (ΔprdB) to grow in the presence of commensal *Clostridia* species (Lopez et al., 2020). The authors found that some *Clostridia* species decreased the advantages *C. difficile* obtained by proline fermentation, while other *Clostridia* species increased these advantages (Lopez et al., 2020), indicating that other *Clostridia* species could alter *C. difficile* proline metabolism. However, this study was performed in batch culture without the presence of other members of the gut microbiota (Lopez et al., 2020). We hypothesized that *C. difficile* proline fermentation could be important for growth in our MBRA communities, which contain *Clostridia* species as well as members of other gut microbiota (Auchtung et al., 2020; Robinson et al., 2014).

To test this hypothesis, we obtained the wt and Δ*prdB* mutants used by Lopez et al. in their study (Lopez et al., 2020) and tested their performance in MBRA microbial communities. For these studies, we cultured fecal communities from the four fecal samples shown in Fig. 2.1(b) to have colonization resistance to *C. difficile* (FS228, FS235, FS515, FS685) and two fecal samples (FS133 and FS583) shown to be susceptible to *C. difficile* infection in Fig. 2.1(b). All six fecal samples were treated with clindamycin as shown in Fig2.3(a) and then challenged with

wild-type (wt) or Δ*prdB* mutant alone or an equal mixture of wt and Δ*prdB* mutant to measure competitive indexes over time. The competitive index is the ratio of wt:Δ*prdB* strains at the end of the experiment compared to the ratio of strains at the beginning. Competition experiments can be used to show decreased fitness of mutants that cannot be detected when mutant strains are cultured alone. Competition experiments were limited to FS133, FS515, FS583, and FS615. From these studies, we saw that across all fecal communities combined, Δ*prdB* mutants declined significantly compared to the wt strain by day 3 in culture (Fig. 2.3(b)-(c)) and also had a competitive disadvantage over time (Fig. 2.3(d)-(e)). However, there were some variations across fecal samples. All four fecal samples that were resistant to *C. difficile* colonization in the absence of clindamycin treatment (Fig. 2.1(b)) had significantly lower growth of Δ*prdB* mutant compared to wild-type on day 5 of infection (Fig. 2.3(l)), whereas the two fecal samples that do not have colonization resistance show no significant differences in levels of wild-type or mutant strain when cultured individually. Somewhat surprisingly, Δ*prdB* has a competitive disadvantage on day 5 in two of three replicate cultures in FS133 (CI on day 5=2.5, 0.5, and 17.8), indicating that there could be some dependence for proline on persistence in these communities. (wt and Δ*prdB* strains were not detected in three replicates of FS583 by day 5 of infection as they had washed out). These results suggest that proline metabolism can be a nutrient niche occupied by *C. difficile* in some MBRA communities.

To test whether the Δ*prdB* mutant is actually disadvantaged due to being unable to metabolize proline, we constructed a tetracycline-inducible copy of *prdB* on a self-replicating plasmid to complement Δ*prdB* as described in Methods. Plasmid stability testing indicated that this plasmid was maintained in 66.7% of cells after 12 generations without antibiotic selection, indicating that it may be stable enough to persist in MBRA cultures. We tested growth of

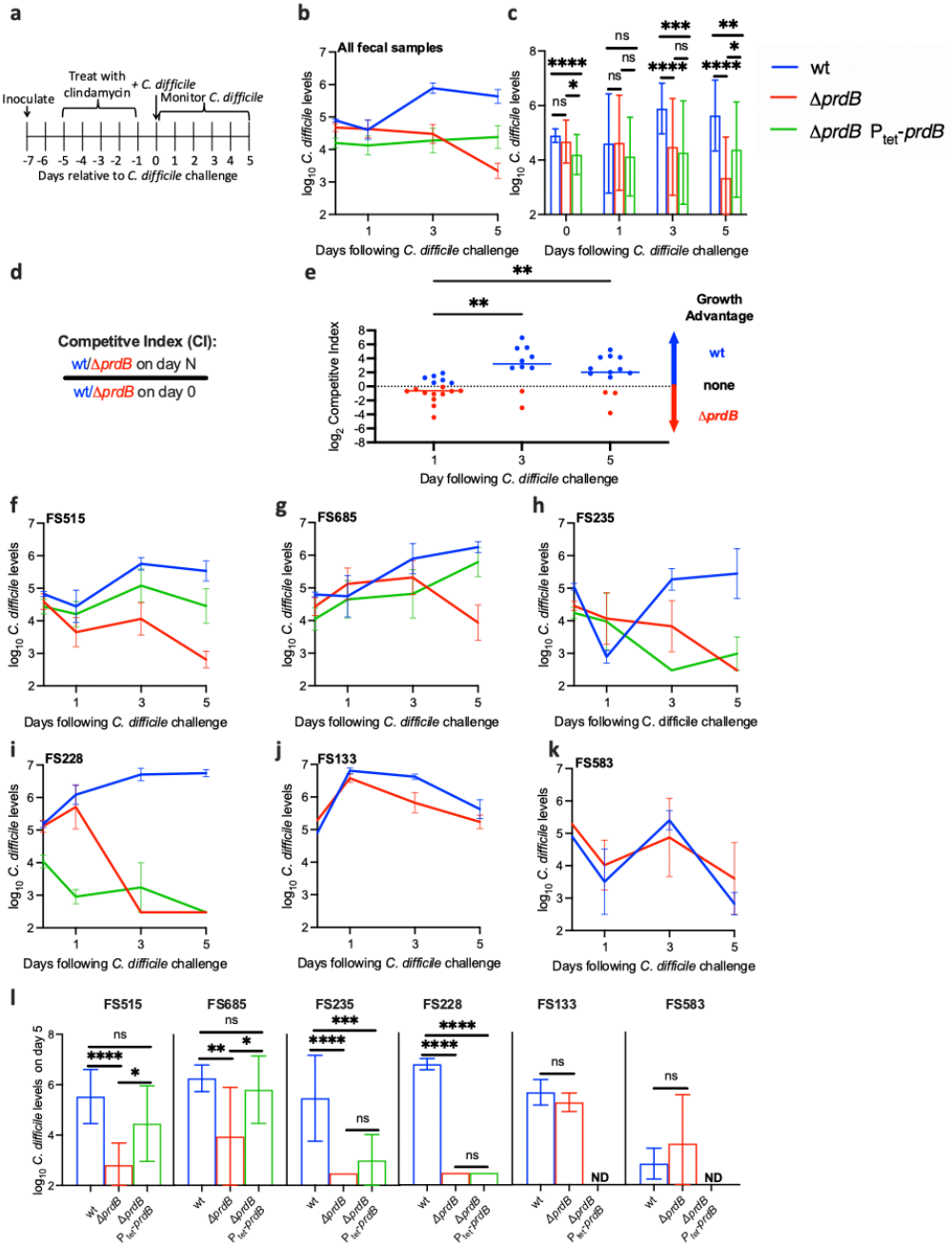

Figure 2.3. Proline metoabilsm is required to colonzie a subset of MBRA communities. a Overview of MBRA time course used to measure growth of wt, Δ*prdB,* and Δ*prdB* strain expressing *prdB* from tetracycline-inducible promoter (P_{tet}-*prdB*) strain of *C. difficile* 196 in different fecal communities. **b-c**. Growth of wt, Δ*prdB*, and/or Δ*prdB*/P_{tet}-*prdB* strains in all six fecal samples shown in panels f-l. **b.** Line represents mean ± SEM. **c.** Bar represents mean ± SD **d-e.** Competitive Index of wt and Δ*prdB* strains co-infected into FS133, FS235, FS515, FS583, or FS685was calculated as shown in (**d**) and plotted in (**e**). Number of replicates shown in Table 2.3 **f-k**. Mean growth of wt, Δ*prdB*, and/or Δ*prdB*/P_{tet}-*prdB* ± SEM over time in (f) FS515, (g) FS685, (h) FS235, (i) FS228, (j) FS133, (k) FS583. Number of replicates shown in Table 2.X in Methods. **l.** Bar represents mean ± SD levels of *C. difficile* in fecal communities indicated on day 5 of infection. Statistical significance was determined by Student's t-test with Welch's correction for unequal variances; *, $p<0.05$; **, $p<0.01$, ***, $p<0.001$; ****, $p<0.0001$.

complemented strains in four fecal communities, FS515, FS685, FS235, and FS228. In FS515 and FS685 communities, we saw that there was significantly increased growth of complemented strains compared to Δ*prdB* strains and that modest differences between wt and complemented strains were not statistically significant. In FS235 and FS228 strains, there were no significant differences between Δ*prdB* and complemented strains as both strains were detected at low levels. Because *prdB* is expressed from a plasmid that is not 100% stable it is possible that plasmid loss was higher in these experiments. Another possibility is that these communities inactivate the anhydrotetracycline used to induce gene expression. Future studies should test a copy of the gene integrated into the chromosome and expressed under its native promoter to rule out these possibilities. However, the results show that at least in some MBRA communities, loss of PrdB function is responsible for decreased growth.

2.4.4 Commensal Clostridia do not restore colonization resistance

In Chapter 1, we described that gnotobiotic mice colonized with *Clostridium bifermentans*, a Stickland fermenter, before introduction of *C. difficile* developed milder symptoms and had a significantly lower death rate (Girinathan et al., 2020). However, in the same study, disease severity of CDI in gnotobiotic mice colonized with *Clostridium sardiniense*, a butyrate-producing Clostridia, was enhanced (Girinathan et al., 2020). Since *C. bifermentans* share a similar metabolic profile with *C. difficile* while *C. sardiniense* does not, colonization of these strains in gnotobiotic mice conditioned the nutritional environment differently, resulted in one that disadvantage *C. difficile* growth and the other favored *C. difficile* growth, with a significant difference of proline concentrations in *C. sporogenes*-colonize mice (Girinathan et al., 2020). As this study was completed *in vivo* without complex gut microbiome, we were interested in if similar effect would be observed in MBRA communities.

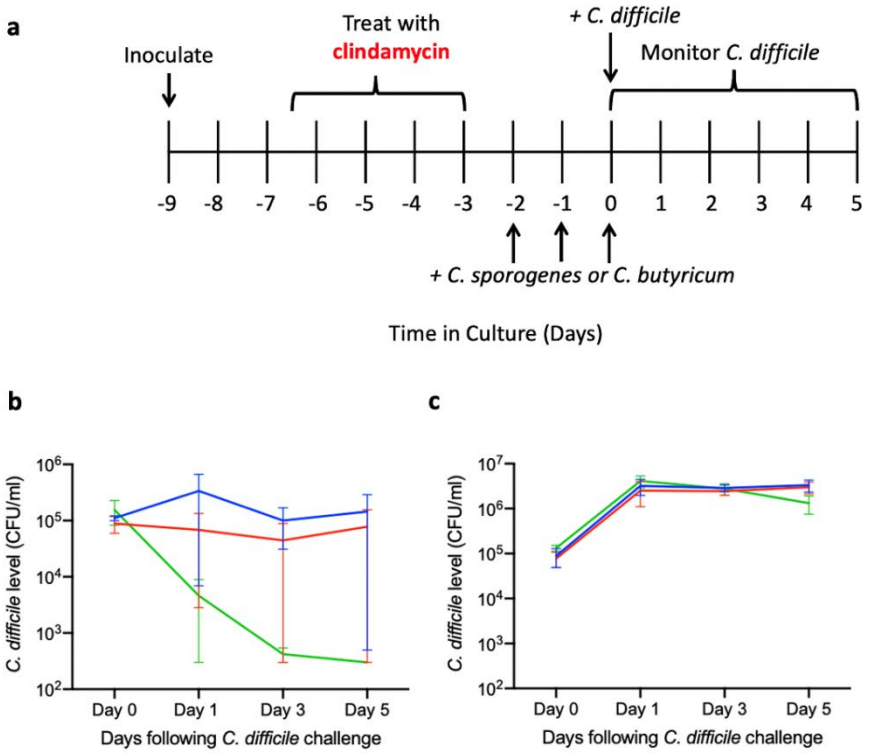

Figure 2.4. Commensal Clostridia do not restore colonization resistance. a. Overview of MBRA time course used to measure growth of wt strain of *C. difficile* 196 in fecal communities with pre-introduction of *C. sporogenes* or *C. butyricum* or without pretreatment. **b-c.** Mean growth of CD196 over time ± SEM in (b) FS515, (c) FS685. N = 3 replicates for each condition.

In our experiment, instead of *C. bifermentans* and *C. sardiniense*, we used another Stickland fermenter, *Clostridium sporogenes*, and *Clostridium butyricum*, a non-Stickland fermenter that can produce butyrate that used as a probiotic in Asia (Chen et al., 2020). We used these strains because both have been tested for use in humans. To complete this experiment in MBRA, we altered the timeline of MBRA operation by adding three days of dosing culture of *C. sporogenes* or *C. butyricum* prior to challenge of wt strain of *C. difficile* 196 and *C. difficile* level was monitored till day 5 after challenge. Details are shown in Figure 2.4(a) and described in Methods. From the results shown in Figure 2.4 (b)&(c), we can see that in two tested fecal communities, pre-colonization of *C. sporogenes* or *C. butyricum* in FS515 community enhanced *C. difficile* level compared to group without pre-colonization; while in FS685 community, *C.*

difficile level in all three groups stayed at high level till the end of observation period. Since *C. difficile* level was not decreased and even elevated in *C. sporogenes* colonized group, introduction of nutrient competitor of *C. difficile* may not exert the same effect within complex communities that have a more complicate nutrient competition relationship among all members than in monocolonized conditions.

2.5 Discussion and Conclusions

In this study, we hypothesized that amino acids, especially proline, could be one of the open nutrient niches important for *C. difficile* colonization in susceptible communities cultured in human fecal communities. To test our hypothesis, we first compared *C. difficile* growth in spent medium supplemented with complete medium containing all requirements required for *C. difficile* growth and incomplete medium that lacked either amino acids, sugars, or yeast extract. Across all three fecal samples tested, we found that *C. difficile* growth was significantly decreased when amino acid availability was limited, which is one reason that we focused on amino acid metabolism in MBRA communities. However, limitation of other categories of nutrients also led to decreased *C. difficile* growth in some of tested groups, indicating that *C. difficile* metabolism of sugars may also be a secondary nutrient niche important for growth in fecal communities and that there may be more than one nutrient niche available in some disrupted communities.

The data we collected from testing requirements of proline metabolism for *C. difficile* colonization in MBRA communities also supports the idea that *C. difficile* colonizes different nutrient niches across fecal communities. When we compared levels of wild type, *ΔprdB* mutant (unable to ferment proline), and Δ*prdB* P_{tet}-*prdB* (*ΔprdB* mutant with restored ability to metabolize proline) in several MBRA communities we found that proline mentalism is only

required in colonizing some communities while not in the others. Specifically, we found that proline metabolism was not required in communities that were susceptible to *C. difficile* colonization in the absence of antibiotics, suggesting that competition for proline may be one important mechanism for inhibiting *C. difficile* colonization in our MBRA model. As several previous studies point to proline metabolism as potentially important niche during human infections, these results provide further support for use of this model to understand *C. difficile* colonization dynamics and identify potential treatments.

We tested whether treating communities with *Clostridium sporogenes*, which shares a similar nutrient requirement with *C. difficile*, prior to *C. difficile* challenge would inhibit *C. difficile* colonization. However, we found that *C. difficile* colonization remained at high levels or were enhanced by treatment with *C. sporogenes*. Since *C. difficile* levels were not decreased and even elevated in *C. sporogenes* colonized group, introduction of a potential nutrient competitor of *C. difficile* may not exert the same effect within complex communities that have a more complicated food web among all members than in mono-colonized conditions in mice. Alternatively, *C. sporogenes* and *C. difficile* may metabolize different nutrients in our *in vitro* culture conditions. In support of this, we found that *C. sporogenes* and *C. difficile* could persist at equally high levels when co-cultured together in MBRAs in the absence of a complex community (data not shown). A third possibility is that levels of *C. sporogenes* were too low to have an effect. Future studies could test whether introduction of *C. sporogenes* alters amino acid availability and whether colonization with other proline metabolizing organisms such as *Lactobacillus rhamnosus* GG (Kim et al., 2021) or combinations of organisms would be a better approach to inhibit *C. difficile.*

Taken together, our results suggested that amino acids could be an important niche for *C. difficile* colonization in some microbial communities while not in the others. Differences in nutrient niche between human fecal communities is consistent with what has been observed in mice treated with different antibiotics (Jenior et al., 2017) and is likely reflective of differences observed across human patients. The open nutrient niches for *C. difficile* in susceptible communities are probably more different from one another and further studies are needed to investigate these different nutrient niches and mechanisms for *C. difficile* colonization. Future studies based on this work could use analysis of sequence and metabolomics data from communities combined with compound and pathway enrichment analysis and the susceptibility of those communities to *C. difficile* to begin to develop predictive models for the important nutrient niches in a specific community and react accordingly to assist in preventing or treating CDI.

2.6 Acknowledgements

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