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Prevention and Cure of Murine C. difficile Infection by a Lachnospiraceae Strain

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by

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ABSTRACT

The goal of this thesis project was to understand how fecal microbiota transplant treats *Clostridioides difficile (C. difficile)* infection, potentially inspiring more effective noninvasive therapies to restore the microbiome as a means of mitigating the disease burden caused by this pathogen. Accordingly, I sought to identify taxa that provide colonization resistance against *C. difficile*. I devised a gnotobiotic ASF (Altered Schaedler Flora) mouse model of CDI that provided a tractable and defined platform for studying microbiota's role in CDI. Challenge of ASF mice with 10^5 spores of hypervirulent *C. difficile* strain VPI10463 (acute challenge) led to complete mortality within three days; challenge with 10^2 spores resulted in low mortality and mice that remained chronically infected by *C. difficile* (chronic CDI model) and, concomitantly, chronic CDI symptoms. Furthermore, I found a previously described *Clostridia* preparation (Kim et al., 2017) protected ASF mice from acute challenge, and led to cessation of VPI10563 shedding and resolution of CDI symptoms in chronically infected ASF mice. Sequencing-based microbiome analysis revealed an association between *Lachnospiraceae* microbes and recovery. Bulk culture of the *Clostridia* preparation was used to generate a *Lachnospiraceae* consortium that protected mice against acute and chronic CDI. Deep sequencing found that recovery of chronic CDI mice strongly corelated with the appearance of a Lachnospiraceae species, namely Uncultured Bacteria and Archaea (UBA) 3401 a bacterium, which, as its name implies, was only known to exist as from databases of shotgun sequencing. While iterative removal of non-UBA3401 microbes from the *Lachnospiraceae* consortium enabled *in vitro* isolation of PCR positive-UBA3401 colonies, subculture of such colonies failed to propagate UBA3401. Thus, I performed iterative limiting dilution fecal transplants of the *Lachnospiraceae* consortium resulting in generation of a consortium predominated by UBA3401, albeit accompanied by a few other microbes (UBA3401 consortium). Inoculation of mice with the UBA3401 consortium was

tracked longitudinally. Sequencing and PCR testing showed UBA3401 became detectable 3 days post inoculation and peaked in absolute and relative abundance at 18 days post inoculation. The UBA3401 consortium-colonized mice and age matched ASF controls underwent acute challenge with VPI10463. Severe CDI followed resulting in complete mortality of the control group, however UBA3401-consortium mice suffered no mortality and demonstrated minimal illness, showing that despite the simple, defined composition of the UBA3401 consortium, significant protection was retained. A concentrated chloroform extract of UBA3401 mouse feces was shown to result in reduced in-vitro growth of VPI10463 compared to ASF feces chloroform extract. Genomic sequencing of UBA3401 uncovered a probable biosynthetic gene cluster (BSC) responsible for production of a Thiopeptide bacteriocin. Canonic thiopeptide BSC genes, including the critical YCAO enzyme, were predicted along with adjacent genes for transport and localization (Vinogradov & Suga, 2020). The UBA3401 genome was predicted to encode many genes involved with quorum sensing, potentially regulating bacteriocin production (M. Kleerebezem et al., 1997), suggesting that a threshold of UBA3401 is required to induce thiopeptide production. Collectively, my results provide a more tractable model to study CDI and yield UBA3401 as potential means of providing microbiota-mediated CDI resistance.

INDEX WORDS: *Clostridioides difficile*, intestinal microbiota, fecal microbial transplant, bacteriocin

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DEDICATION

To everyone who has encouraged me to think, to question why, to seek answers and not give up on my journey to better myself, thank you. To my colleagues, comrades and guides in this journey, thank you, this paper is dedicated to you. Without the aid and instruction, I received from my mentors at Georgia State University, this study would not have been possible. To the many excellent professors I learned from while a student, I thank you, for without you I could not have understood the concepts behind this study. To all the brilliant and kind minds I worked alongside while a graduate student, especially the Assistant Research Professors, post-doctoral scientists and laboratory managers of the Gewirtz lab, I thank you for without your advice and expertise to show me how to carry out research or collaborate with, it would not have been possible to plan and carry out the experiments needed for this project to succeed. To my committee members, many professors who had instructed me early on in my graduate program, I thank you, for without your edifying instruction and advice on how I may better myself as a researcher and better my project and better my ability to convey what I have discovered, this project could not have reached a state fit to publish. To my advisor, mentor and PI Andrew Gewirtz, I thank you, for without your guidance at every step of the way and the aid of a leading expert in the field of interactions between microbes and their hosts, I could not have fully understood the significance of our project, nor seen and understood how powerful symbiotic microbes can be. You have been a superb leader and coach that understands his students' strengths and weaknesses and have been very patient.

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Introduction

Many of the same principles involved in the invasion and overrun of an ecological niche by invasive pests are involved in opportunistic diseases that claim tens of thousands of lives each year. Of these disease-causing opportunistic pathogens none is more archetypal than *Clostridioides difficile (C. difficile)*: an obligate anaerobic gram-positive spore-forming microbe notable for its toxigenic properties and its prominence as a healthcare-acquired pathogen (Sinnathamby et al., 2023). *C. difficile* was first described by Ivan Hall and Elizabeth O'Toole as a component of neonatal human stool that faded away in the weeks following birth. Although not disease-causing in neonates, acellular filtrate of broth cultures containing *C. difficile* – originally named *Bacillus difficile* due to the great difficulty in growing a pure culture – injected intraperitoneally was found to be remarkably lethal to small rodents (Hall & O'toole, 1935). Both the inability of *C. difficile* to cause disease and to persist in healthy human hosts and the remarkable virulence of its TcdA exotoxin suggested by these early observations, while not appreciated at the time, would demonstrate the key traits of the disease. In the absence of shocks to the microbiota, *C. difficile* encounters an unassailable ecosystem, its niches already taken by competitors with superior fitness thwarting invasion – via the principle of colonization resistance (Caballero-Flores et al., 2023). However, if the host microbe is perturbed through an external shock, such as antibiotics, voids left in the host's microbial ecosystem open niches for *C. difficile.* The centrality of microbiota in protecting from *C. difficile* has increasingly been appreciated in therapeutic interventions to restore the lost colonization resistance. Consequently, this project sought to shed light on which taxa play a predominant role in *C. difficile* colonization resistance and suggest possible mechanisms that can be elucidated upon in subsequent studies.

Historical Background: Discovery of *C. difficile* and it's Pathogenesis

The age of antibiotics, spurred by breakthroughs from brilliant researchers spanning from Paul Elrich to Alexander Fleming, yielded immense benefit for human health. However, the advent of antibiotics has been followed by the proliferation of myriad diseases. Notably patients under prolonged antibiotic regimens would frequently become ill, consumed by opportunistic pathogens blooming from the antibiotic-created voids in the host's microbiota. Indeed, gastrointestinal illness, striking the most microbe rich system of the body, represents the most common adverse effect of antibiotics. Such illnesses, ranging from diarrhea to lethal colitis, with severe manifestations such as pseudomembranous colitis – historically with mortality rates in excess of 50% – are common consequences of treatment with broad spectrum antibiotics. Notably Clindamycin's propensity to cause gastric disease became apparent within years of its discovery, with a staggeringly high 10% of clindamycin users in early studies developing the condition following use (Tedesco et al., 1974). Although initial attempts at isolating a causative pathogen were unsuccessful, the discovery of a toxin in animal models of CDI was suggestive of antibiotics clearing a path for a toxin-producing pathogen (Tedesco et al., 1974).

In retrospect, Hall and O'Toole's discovery of *C. difficile* and its toxigenic properties presented a strong candidate for the causative pathogen. However, because of *C. difficile*'s apparent inability to fulfill Koch's first and third Postulates - *C. difficile* was found in the stool of healthy neonates, and seemingly was non-pathogenic to adults and animal models under typical conditions – that would relegate it to medical obscurity for decades. Progress would come through the improved cultivation methods and characterization of toxigenic properties by Hafiz, easing one of the principal constraints for studying *C. difficile* (Green, 1974; Hafiz, 1974). These advances would lead to a breakthrough in understanding the etiology of CDI in the form of a

systematic effort to identify the causative pathogen (Bartlett et al., 1977). Through concentrating and filtering the cecal material of hamsters that had succumbed to antibiotic induced pseudomembranous colitis, Bartlett obtained a crude form of *C. difficile* toxin. The injection of toxin into healthy hamster ceca recapitulated CDI, confirming that the extract contained the previously reported toxin. Elegantly utilizing the tools available in a hospital of the time, Bartlett et al. attempted to neutralize the toxin with antiserums, thereby suggesting the taxa of the toxicogenic bacteria. Only gas gangrene antitoxin possessed activity against the toxic extract, suggesting a Clostridia served as the causative pathogen. Intensive efforts at culturing Clostridia microbes from the infected mouse ceca and utilizing adverse conditions to select for clostridia, such as heat shock, since Clostridia endospores are known to be heat resistant, led to recovery of multiple candidates. Supernatant from the resultant broth cultures was subsequently injected into the cecum of mice, one culture supernatant was able to replicate the effect of the crude toxin and as predicted, was also neutralized by gas gangrene antitoxin, thereby utilizing the metabolites yielded by the pathogen and its colonies as an intermediate to reproduce Koch's postulates (Bartlett et al., 1977).

Pathophysiological traits, such as cytoskeletal compromise, along with no apparent pathogen had made pseudomembranous colitis widely suspected to be a viral disease (Green, 1974). These traits could be replicated in in-vitro cell cultures; however, no virus could ever be recovered. Much like the hamster model, treatment of cell cultures with Clostridia antiserum prevented the cytoskeleton damage characteristic of the disease. Repeating the in-vitro cytotoxicity assay with samples obtained from patients with pseudomembranous colitis found again that clostridia antitoxin could prevent toxicity, further linking clinical cases with the clostridium bacteria. The isolate designated Clostridium BVBA 17 HF1-9 (named after the

Boston Veteran's Hospital most of the research was carried out in) was presumed to be *C. difficile(Bartlett et al., 1977)*. Subsequent clinical studies by Bartlett confirmed *C. difficile* to be present in all patients suffering from pseudomembranous colitis, with acellular extracts of the samples injected into hamster ceca, or in vitro cell cultures, resulting in the same lethality seen in the mouse-isolated *C. difficile*. As predicted, clostridia antitoxin neutralized toxicity in the animal and tissue culture model. Notably, toxin was found in some patients with milder adverse reactions to antibiotics, such as diarrhea; and *C. difficile* toxin was found in patients who had received other commonly used antibiotics including β-Lactams (Bartlett et al., 1978). Thus, not only was *C. difficile* established as the causative pathogen for pseudomembranous colitis but became apparent that its manifestations existed in a spectrum that encompassed many of the more moderate iatrogenic results of antibiotics. Many of the techniques used to uncover *C. difficile*'s role, such as the cytotoxicity assay, would go on to become mainstays for diagnosing CDI (Bartlett et al., 1978).

C. difficile Pathophysiology

A spore forming obligate anaerobe, *C. difficile* is very capable of infecting new hosts, despite its inability to survive as a vegetative microbe in the presence of oxygen. From inside its natural reservoir – the mammalian intestine – sporulation is marshaled by the transcription regulator Spo0A, which crescendos in activity in a vegetative cell; after several cycles of division and nutrient depletion it induces asymmetric division producing the smaller, infectious endospore form of the bacteria (Fujita & Losick, 2003). The *C. difficile* spore is broadly similar to other members of its phylum, despite possessing significant distinctiveness at the molecular level. An outer hydrophobic exosporium provides *C. difficile* with the ability to adhere to surfaces and also possesses cytotoxicity against phagocytosing macrophages (Paredes-Sabja et

al., 2012). Combined with the inherent resistance to antibiotics (Baines et al., 2008), chemicals, and heat, the endospore form of *C. difficile* is well suited to survive adverse environments long enough to reach the intestine of a host (Paredes-Sabja et al., 2014). Sporulation is so vital to the propagation of *C. difficile* that loss of Spo0A will completely abolish its ability to transmit to a new host, and compromises the ability of *C. difficile* to remain in its current host (Deakin et al., 2012).

Once a host small intestine has been reached, the presence of nutrients activates receptors inducing germination, which continues as the pathogen reaches the large intestine where growth begins. In *C. difficile*, the bile salt cholate and its derivatives, along with glycine, serve as the primary germinants allowing the seeds of pathogenic *C. difficile* to realize that they have reached fertile ground and bloom (Paredes-Sabja et al., 2011). Germination receptors once activated utilize a Ca2+-DPA signal transduction pathway to marshal cellular machinery towards germination (Paredes-Sabja et al., 2011). With germination induced, hydrolases and proteases are produced to break down the outer peptidoglycan layer of the spore capsule allowing hydration and metabolic processes and growth to commence. The hardy nature of the *C. difficile* spore frustrates efforts at decontamination, with disinfectants failing to inactivate the endospore form of the pathogen and the endospore's adhesive coat, allowing *C. difficile* to remain on surfaces until they adhere to a host and enter the oral fecal route. Such problems are worsened by the extreme susceptibility to infection from *C. difficile* of antibiotic-treated hosts (Lawley et al., 2010). Attempts at preventing the spread of *C. difficile* are further stymied by the other mammals also serving as a reservoir, indeed, increasing evidence suggests that *C. difficile* is endemic to farm animals – often raised under constant antibiotic regimens – with infected animals constantly shedding infectious spores into water and other environmental sources (Lim et al.,

2020). Ergo infection control at a hospital, even if completely successful, will not eliminate disease reservoirs for CDI.

The most significant cause of disease from a *C. difficile* bloom in a susceptible host can be attributed to the pathogen's production of the TcdA/TcdB exotoxins. First observed by Hall and O'Toole, the toxin was notable for a remarkably low LD_{50} via intraperitoneal injections. Decades later, experiments with partially purified toxin extracts demonstrated its ability to recapitulate CDI, if administered to animals, and the cytotoxic effect upon cells (Green, 1974). Furthermore, the attempts at purifying the toxin through chromatography suggested the toxin's constitution to be binary (Taylor et al., 1981). Contemporary knowledge has affirmed these observations with the *C. difficile* exotoxin now known to be an archetypal example of an AB exotoxin. Organized in a single locus, likely of viral origin, TcdA and TcdB are transcribed to form a binary exotoxin with glucosyltransferase activity. Once endocytosed into host enterocytes via endosome, the acidic environment activates the TcdA/TcdB proteins (Voth & Ballard, 2005). Once active inside a host cytosol, the toxins glycosylate GTPases (Rho, Rac, and Cdc42) compromising enzymes vital to the function of the cytoskeleton (Chaves-Olarte et al., 1997). The loss of GTPases not only compromises cellular signaling, but also leads to the virtual collapse of the cytoskeleton, due to the centrality of GTPases in actin and microtubule function. The dramatic effect that TcdA/TcdB exotoxins have upon the most fundamental aspects of cell biology make them one of the most lethal toxins by moles in in-vitro models (Aktories & Jank, 2015). The binary Tcd exotoxins are so central to the pathology of CDI that mutations leading to hyperproduction of TcdA/TcdB via loss of negative regulators (Warny et al., 2005), or more efficient exotoxin function (Lanis et al., 2012), are believed to be responsible for the hypervirulence of many strains (Janoir, 2016; Merrigan et al., 2010).

The TcdA/TcdB exotoxins are not the only virulence factors present (Voth & Ballard, 2005). Additional toxins are encoded by the *C. difficile* genome; most notably *C. difficile* transferase (CDT): an actin-ADP-ribosylating toxin (McDonald et al., 2005). In a manner analogous to the TcdA/TcdB disabling GTPases, CDT will ribosylate G-actin, preventing elongation of the filament and inducing depolymerization; consequently, derangement of the cellular cytoskeleton is aggravated by the attack upon this second key component (Perelle et al., 1997; Schwan et al., 2009). Furthermore, in-vitro and in-vivo evidence suggests CDT-induced changes to microtubule networks result in protrusions on the affected cell's surface. These protrusions appear to improve *C. difficile's* adherence to its host, thereby prolonging infection (Schwan et al., 2009). As expected, CDT is associated with more severe disease and several hypervirulent strains are known to encode it (McDonald et al., 2005), although presence of CDT is neither necessary nor sufficient for a hypervirulent phenotype (Voth & Ballard, 2005). Additional *C. difficile* traits facilitate its invasion of a host: chemical modification of its cell wall via deacetylation of GlcNAc peptidoglycan allows *C. difficile* to be inherently resistant to lysozymes and peptidoglycan hydrolysis, while fibronectin-binding protein and the network of proteins that compose *C. difficile*'s surface layer (S-layer) allow adherence to a host. The S-layer and it's key constituent Surface Layer Protein A (SlpA) are critical to *C. difficile's* invasion of a host as SlpA is not only necessary for *C. difficile's* attachment to host cells (Calabi et al., 2002), but also antibodies targeting SlpA are sufficient to prevent attachment in in-vitro model (Merrigan, 2010). Additional functions of S-layer proteins include proteases which target the host (Vedantam et al., 2012). Notably, purified SlpA in itself is sufficient to compromise tight junction expression while inducing TNF-α, IL-1β and IL-6 cytokine production in HT-29 cell

cultures, suggesting the *C. difficile* surface proteins in and of themselves are toxic to the intestinal epithelia (Noori et al., 2022).

If host microbes that can exclude *C. difficile* are not present, then *C. difficile* can use its aforementioned traits to invade, germinate in, adhere and proliferate in a host, releasing exotoxin. The disruption of intestinal epithelial cytoskeleton and junctions, inevitably compromises the intestinal barrier function (Pothoulakis, 2000). Consequent intestinal permeability directly results in diarrhea. Due to fluid loss accompanying the outflow of electrolytes and aggravated inflammation as microbial products translocate into the host, it promotes a feedback cycle of worsened barrier function and increasing inflammation (Poxton et al., 2001). Necrosis of intestinal epithelial cells results in lesions on the surface epithelium, initially only presenting as nonspecific diffuse colitis. As leukocytes translocate into the lumen and the products of dead cells and fibrin accumulate, pseudomembranous plaques are formed (Farooq et al., 2015). As necrosis expands, inflammation extends deeper into the crypt, with crypt lesions developing and pseudomembranes growing to become confluent, resulting in large pseudomembenous plaques visible under endoscopy. At its most severe, necrosis and injury will extend to transmural depth and life-threatening fulminant colitis results. (Reggiani Bonetti et al., 2021). Gross anatomical signs include colon wall thickening, submucosal edema, and with increasingly severe manifestations, colonic dilation or perforation and ascites (Farooq et al., 2015). The spectrum of clinical manifestations largely corelates with the aforementioned anatomic findings, with mild CDI denoted by diarrhea without elevated leukocyte counts, moderate CDI with diarrhea and elevated WBC, severe also showing fever and signs of major colon or organ injury, and the most severe cases shock and manifestations of fulminant colitis such as toxic megacolon.

Epidemiology, Treatment and Research

Unsurprisingly, due to the significant virulence of *C. difficile*, approximately 29,000 Americans diagnosed with CDI will die within 30 days even while under the care of contemporary medicine (Fu et al., 2021; Guh et al., 2020) Although initially uncommon, Hypervirulent strains, denoted by increased TcdA/TcdB activity such as 027/NAP1/BI, have become predominant (Janoir, 2016; Song & Kim, 2019). Despite improved infection control, cases remain high, at half a million per annum (Fu et al., 2021; Gerding et al., 2008; Guh et al., 2020). Modest reductions in the incidence of iatrogenic CDI have been offset by rising cases of community acquired CDI, which confound traditional methods of controlling CDI (Fu et al., 2021). Although broad spectrum antibiotics remain the primary cause of *C. difficile* infections other risk factors including colorectal surgery (Aquina et al., 2016), chemotherapy or immunosuppression (Napolitano & Edmiston, 2017), and even severe infections or injury that disrupt the intestinal equilibrium may result in lethal CDI as a sequelae even if antibiotics are not used (Halvorson et al., 2011). Indeed, more than a third of community acquired CDI cases occur in patients who have not taken antibiotics (Fu et al., 2021; Guh et al., 2017). Non-antibiotic risk factors for community acquired CDI include GI disease and PPI use, along with cardiovascular and kidney disease (Guh et al., 2017). Curiously, IBD patients report alarmingly high rates of asymptomatic carriage at over 8.2%, possibly as a consequence of the profound dysbiosis present in IBD patients (Fu et al., 2021; Gevers et al., 2014); although it is also possible “asymptomatic” carriage of *C. difficile* aggravates IBD. It is this profound dysbiosis in CDI patients that allows *C. difficile* to persistently colonize or reinfect CDI patients, with up to 30% of patients going on to relapse after an initial infection (Song & Kim, 2019).

Initial treatment for uncomplicated CDI still is centered around clearance of the infection through antibiotics and attempts to prevent relapse by discontinuing the triggering antibiotic. Metronidazole remains a favored treatment for mild CDI, although its potential for neurotoxicity disallows prolonged use for relapsing disease (Cohen et al., 2010; Cole & Stahl, 2015). Vancomycin remains the preferred first line therapy for moderate and severe CDI. It can be used as a colon irrigation, or a pulsed regimen administered over the course of weeks for relapsing CDI. Although often effective, both Metronidazole and Vancomycin possess a failure rate in excess of 30% (Cole & Stahl, 2015). Substitution of the typical antibiotic regimens with the newly approved narrow spectrum antibiotic Fidaxomicin, less disruptive to the microbiome than Vancomycin, appears to cause a relapse at half the rate of Vancomycin, but still will not prevent a profoundly dysbiotic host from remaining susceptible to reinfection (Louie et al., 2011; Song & Kim, 2019), and its very high cost limits its use as a first line therapy (Cole & Stahl, 2015). Probiotics have been explored as adjunct therapy to prevent relapse or reinfection and present a generally safe and cost effective intervention but the available species have shown very limited efficacy to the extent that their value as an intervention has been questioned (Cohen et al., 2010; Cole & Stahl, 2015; Seltman, 2012), with fungal probiotics being contraindicated in critically ill patients due to risk of fungemia (Butler et al., 2011; Seltman, 2012). Nevertheless some species, including *Bacillus coagulans*, appear to possess superior efficacy to other commercially available species (Fitzpatrick, 2013).

Surgical intervention remains standard treatment for life threatening cases of colitis; with the limitations of antibiotic treatment, along with the proliferation of hypervirulent strains, resulting in up to 10% of patients progressing into relapsing CDI or fulminant colitis (Neal et al., 2011; Seltman, 2012). Although considered for all patients with worse than moderate CDI,

surgical resection is primarily employed with patients suffering from complicated CDI where colon rupture and shock are an imminent risk; indeed the risk of death for hemodynamically unstable patients treated with emergent colectomy appears to be reduced, compared to critically ill patients who did not receive surgical intervention (Lamontagne et al., 2007). Nevertheless, removal of colon is not only a debilitating intervention, but the risk of death remains high at roughly 50% (Seltman, 2012). Attempts at limiting the amount of tissue removed via subtotal colectomy or salvaging as much colon as possible through diverting Ileostomy and colon lavage have been utilized with the benefit of reduced disability, nevertheless fulminant CDI remains a condition with a high risk of morbidity and mortality (Neal et al., 2011; Seltman, 2012).

The discovery of a pathogenic bacteria that bloomed following antibiotic use and resulted in pseudomembranous colitis suggested the body's commensal flora played a role in preventing CDI. Evidence in support of this theory already existed from the attempts of Stanley Falkow to utilize autologous FMT to protect patients from antibiotic exposure, and an independent case series utilizing fecal enema as a rescue therapy for patients with refractory CDI under the rationale competing microbes would displace the pathogenic staphylococci then suspected of causing pseudomembranous colitis (de Groot et al., 2017; Eiseman et al., 1958). Although effective, research into FMT for treatment of CDI stagnated as the cytotoxicity of *C. difficile* lead to the presumption of a viral etiology for pseudomembranous colitis (Borody et al., 2015; Collins, 1960; de Groot et al., 2017). With the discovery of the causative agent of pseudomembranous colitis, a resurgence in attempting to restore colonization resistance took place and more case reports noting the efficacy of fecal enema as a rescue therapy emerged (Bowden et al., 1981). Positive results continued with further case series (Schwan et al., 1983, 1984) thus inspiring attempts at more targeted probiotic treatment, most notably with a non-

toxigenic strain of *C. difficile* that helped diminish levels of virulent *C. difficile* and its morbidity (Seal et al., 1987). The microbial interventions, along with use of immunoglobin antitoxins or cholestyramine to bind *C. difficile* exotoxins represented the most promising nonsurgical approaches to combating CDI of the late 20th century (Lyerly et al., 1988). The proliferation of more virulent subtypes of *C. difficile* and the increasing failure rate of antibiotic interventions in the early 21st century drove additional interest in FMT, and although data was still limited to case series, the efficacy of FMT had become abundantly clear with a success rate estimated at close to 90% (Kassam et al., 2013), a figure borne out by RCT carried out as FMT became the mainstream treatment for recalcitrant CDI in the second decade of the 21st century (van Nood et al., 2013).

The transplant of the entirety of a host's microbiome, however, is not without significant risk (Marcella et al., 2021): host microbes are heterogeneous and impart myriad effects upon host phenotype, many potentially negative such as from taxa associated with atherosclerosis, cancer, obesity and even gut-brain axis diseases such as Parkinson's (Cirstea et al., 2020; Deakin et al., 2012; Zhu et al., 2022). Ergo, transfer of all microbes within a host can, and has resulted in short term and long term adverse events, with serious adverse effects estimated to occur at a rate of 1.4% (Alang & Kelly, 2015; Baxter et al., 2015; Marcella et al., 2021). Indeed, transmission of pathogens (Zellmer et al., 2021) some of which have resulted in patient deaths, in spite of attempts to screen donor feces, demonstrates the risks of transferring a large, undefined community of microbes (Michailidis et al., 2021; Solari et al., 2014). A 2019 FDA advisory, due to the spread of drug resistant *Escherichia coli* via FMT resulting in at least one confirmed fatality, provides a concrete demonstration of this risk. Thus, even if FMT represents a superbly effective method of clearing CDI by restoring the microbes responsible for colonization

resistance, the invasiveness, its cost, and potential risk of the procedure prevent it from serving as a first line therapy.

Although commercially available probiotics used as adjunct therapies show minimal efficacy, the concept of administering a narrowly targeted, safe, and defined set of microbes to a host is attractive. A growing body of animal model data suggests certain taxa can exert disproportionate colonization resistance against CDI (Greathouse et al., 2015; Maslanka et al., 2020). However, the mechanisms and taxa responsible for the microbiome remain poorly characterized. A significant obstacle to the study of individual microbes and mechanisms in animal models is the extreme variability of the mouse microbiome, which has translated into radically different susceptibilities to antibiotic induced CDI (Etienne-Mesmin et al., 2018; Maslanka et al., 2020). A solution to these concerns is offered in the form of the Altered Schaedler Flora (ASF) gnotobiotic mouse: ASF mice are colonized by an eight species consortium composed of: Parabacteroides goldsteinii, Eubacterium plexicaudatum, Schaedlerella arabinosiphila, Pseudoflavonifractor sp., Clostridium sp., Mucispirillum schaedleri, Ligilactobacillus intestinalis, Lactobacillus murinus (Wymore Brand et al., 2015). The ASF mouse possesses a more normal intestinal and immune physiology than germ-free mice (Wymore Brand et al., 2015), allowing for a reproducible and representative model. Most attractively, the ASF metagenomes are well characterized, facilitating mechanistic studies due to the baseline metagenome being well defined (Proctor et al., 2022). Additionally, while the ASF consortium lacks the metagenomic complexity of conventionalized mice, full genes for carbohydrate and energy metabolism are present, facilitating the growth of fastidious syntrophic experimental microbes that may lack the metabolic pathways needed to grow in a germ-free host (Proctor et al., 2022).

Thus, the initial goal of this thesis was to investigate the potential of ASF mice to serve as a tractable model of CDI. As I began to affirmatively accomplish this goal, I sought to use this new model to interrogate microbiome taxa that play predominant riles in providing colonization resistance against *C. difficile*. Subsequently, I sought to isolate a defined microbial consortium capable of protecting mice against CDI. As described in this thesis, I have met these goals. The results herein have been reported in a manuscript currently under review. Furthermore, I have performed genomic characterization of the protective consortium. This analysis has revealed potential mechanisms by which the consortium combats *C. difficile* thus providing direction for future studies.

Methods

Mice: *C57BL/6* mice were either maintained in conventional specific pathogen free housing or gnotobiotic isolators, under germfree (GF) or Altered Schaedler Flora (ASF) conditions, as previously described (Chassaing & Gewirtz, 2018). All mice were maintained with autoclaved 5010 rodent diet and autoclaved water. Experiments utilized 6–12-week-old male and female mice, which were caged by sex.

Preparation and titration of *C. difficile*: *Clostridioides difficile*, strain VPI 10463, was cultured under anaerobic conditions provided via an Anerobic Systems AS-580 Anaerobic chamber, using a mix of 90% Nitrogen, 5% Hydrogen, and 5% CO_2 atmosphere. A single colony was selected and grown in Brain Heart Infusion (BHIS) broth (Millipore cat no. 53286 and Y1625) and a week was allowed to elapse. Broth media turbid with VPI was plated in large volumes (250 ml) over BHIS agar plates; resultant VPI colonies were allowed to grow for two weeks by which time the stationary phase of the microbial cycle will have been reached and sporulation shall have occurred in large numbers. Colony vegetation on the plates was subsequently scraped into a conical tube PBS suspension. Following 15 minutes of centrifugation at 3000g the spore pellet is subsequently washed with sterile PBS, resuspended and heated

at 70C for 15 minutes. Quantification of spore solution CFU is carried out via serial dilution on taurocholate-cefoxitin-cycloserine-fructose agar (TCCFA) plates chosen for their ability to exclude microbes other than *C. difficile* and germinate *C. difficile* spores to ensure only viable VPI 10463 spores were counted (Edwards et al., 2013). Spores were diluted serially to generate desired infectious dose. The lack of a broth form of TCCFA and concerns that antibiotics may interfere with growth assay resulted in the use of BHIS media.

Acute *C. difficile* Challenge: Gnotobiotic mice, ASF or GF, were administered an experimental treatment or vehicle and challenged with 10^5 spores of VPI10463 after 72 hours. Mouse condition was monitored throughout the experiment and shedding, weight, inflammatory cytokines and fecal VPI shedding were monitored for two weeks. Autoclaved cages and food were used throughout the experiment.

Chronic CDI ASF mouse model: 6–8-week-old ASF mice were moved from gnotobiotic isolators to sterilized cages and maintained with autoclaved food/water. *C. difficile* spores were administered at 10 CFU/mouse via oral gavage. Mice were monitored and provided oral rehydration solution to reduce mortality. VPI levels stabilized two weeks after infection allowing for a window of opportunity to test therapeutic interventions under controlled conditions. Mice were monitored for recovery as described for acute challenge model.

DNA Isolation: Fecal or *in vitro* samples were diluted via PBS + 0.1% Tween 20 (100 mg/ml) with 250 µl of the homogenate transferred for extraction. Lysis was performed via bead beating and heating at 64 degrees Celsius for 10 minutes while incubated with 10% sodium dodecyl sulfate, 300 µl of Tris-EDTA Saturated Phenol (Thermo Fisher cat no. 327125000) and 200 µl of Tris buffer (Millipore cat no. 648314). Samples were subsequently centrifuged 5 minutes at 12,000g at 4 degrees Celsius and the aqueous supernatant was collected. Phenol-Chloroform extraction was executed via addition of 450 µl of

Phenol-Tris followed by centrifugation for 5 minutes at 12,000g at 4 degrees Celsius and subsequent collection of aqueous supernatant and incubation with 700 µl of chloroform (Sigma Aldrich cat no. 34854) and centrifugation. A 250 µl aqueous layer was collected and dissolved in 1000 µl of pure ethanol (Decon cat no. 2716) and 30 µl of sodium acetate (Thermo Fisher cat no. AM9740) and stored at –20C for 30 minutes. A DNA pellet was subsequently yielded by centrifugation under 12,000g at 4 degrees Celsius for 30 minutes; pellets are subsequently rinsed with 75% ethanol. DNA purity was tested following resuspension with 100 µl of water. If purity, assessed by 260:280 was unacceptably low, an additional purification via precipitation with ethanol and sodium acetate was performed.

Quantification of VPI10463

Detection and quantification of VPI10463 was carried out via the use of 2.5 µl of sample DNA of acceptable quality under the methods qPCR previously described (Etienne-Mesmin et al., 2018).

Generation of UBA3401 Specific Primers

Partial UBA 3401 sequence in NCBI (NCBI: txid1952033) was compared in bioinformatics via Geneious software (NZ) with other members of the *Lachnospiraceae* and Clostridia taxa. Regions of the UBA3401 genome were analyzed to find sequences not conserved with other bacteria with a UBA3401 sequence homologous to Leucyl/phenylalanyl-tRNA-protein transferase, dissimilar in sequence to all other bacteria assessed. Primers binding inside the unique sequence 2241F (ATGGGAGCCCTGTTGTCCTA) and 2669R (AATCGCCGTTTTACCAACGC) were designed with a 429BP product, specific to UBA3401 being predicted. Testing was carried out with samples confirmed by metagenomic sequencing to be positive for UBA3401 alongside negative controls. The 429BP product was only observed to be present in samples confirmed positive for UBA3401. All samples confirmed by sequencing to be negative for UBA3401, containing *Lachnospiraceae* never produced the 429BP fragment.

Detection of UBA3401

2.5 µl of purified sample DNA was utilized for qPCR with 12.5 µl of Qiagen Quantinova SYBR green master mix (cat no. 208052) per reaction, 8 µl of mq water with 1 µl of 2241F and 1 µl of 2669R primers respectively. The 2241F and 2669R primers are pre-diluted to 10µl/nmol concentration. Amplification was carried out under an initial 10-minute 95° Celsius denaturation followed by 60 cycles of 15 seconds of 95C denaturation and 60 seconds of 60C annealing-extension; followed by a final 60 second 95C and 60 second 60C step. Melt curve was performed under 60 iterations of a 10 second 55C and 0.5 second 95C step. A melt curve peak in the range of 80-81C is suggestive of UBA3401 presence. Confirmation of UBA3401 presence was achieved via gel electrophoresis with a 429BP product corresponding to a unique UBA3401 sequence being highly specific to UBA3401. Ct threshold in UBA3401 positive cycles is inversely correlated with the abundance of UBA3401.

Bulk Culture of Cecal *Lachnospiraceae*

0.2um of filtered 0.2% cystine PBS along with autoclaved *Lachnospiraceae*-selective BHIS agar plates (Millipore cat no. 70138 and Y1625) enriched with 2ml/L Gentamycin (Sigma Aldrich cat no. G1264) and 1ml/L Aztreonam (Sigma Aldrich cat no. PHR1785-500MG) were reduced overnight in a AS580 anaerobic chamber. An ASF mouse was obtained, which had received the simplified consortium 28 days (d) prior and was housed inside an Isocage and euthanized under aseptic conditions. The cecal contents were immediately obtained following euthanasia and placed in 100 mg/ml reduced PBS-Cystine. This suspension was then moved inside the anaerobic chamber and opened to remove oxygen and subsequently homogenized. An additional 1:100 dilution was performed with the cecal homogenate with the diluted solution plated on the *Lachnospiraceae* selective plates and incubated at 36C for 7d. Resulting colonies were harvested in bulk and stored in anaerobic aseptic 30% glycerol PBS with the resulting solution designated the *Lachnospiraceae* Consortium.

Enrichment of UBA3401 relative abundance

Fecal samples were obtained from ASF mice that had received UBA3401. Those samples were subjected to iterative steps of heat treatment at 55C, serial dilution or exposure to solvents including chloroform, and subsequently administered to a Gnotobiotic mouse. 14d post administration, fecal samples were collected from the mouse and presence of UBA3401 was confirmed. Subsequently the mouse was administered kanamycin (Sigma Aldrich cat no. PHR1487) at 50 mg/L via drinking water followed by withdrawal of drug after 48 hours. 72 hours were allowed to elapse before fecal samples were tested to confirm that abundance of UBA3401 – predicted to be inherently resistant to aminoglycosides like most anaerobes (Bryan & Kwan, 1981) – was not depleted. Samples were subsequently collected and used for additional iterations to reduce the number of non-UBA3401 taxa.

Cultivation of UBA3401 Consortium and propagation in ASF host

ASF mice highly enriched for UBA3401 were obtained and euthanized, cecal and fecal samples were quickly collected and placed in anaerobic 0.2% cystine PBS. Samples were homogenized at 100mg/1ml, were serially diluted and streaked onto BHIS plates supplemented with 1mg/L gentamycin and 10ml/L taurocholate 10% inside an AS580 anaerobic chamber. Plates were allowed to grow under anaerobic conditions at 36C for 10d after which individual colonies were harvested and homogenized in 30% glycerol PBS for storage at -80^{o}C. Stock from the colonies was screened for presence of UBA3401. Two samples: a tiro of colonies and a bulk collection of colonies from the same plate tested positive. Both samples were administered to ASF mice held inside Isocages. Fecal samples were collected in one-week intervals and screened to confirm successful UBA3401 engraftment.

Colonization of ASF mice via cecal administration

An ASF mouse donor shown by metagenomic sequencing to be predominant in UBA3401 and shown by qPCR to possess a very high absolute quantity of UBA3401 was euthanized. The donor's cecal materials were immediately collected and resuspended in anaerobic 30% glycerol, 0.1% cystine PBS at 100mg/ml concentration. The cecal contents were gavaged in a short timeframe to maximize delivery of vegetative

microbes. Fecal samples were obtained in 3d intervals to track engraftment and expansion of UBA3401. The presence of UBA3401 was confirmed by electrophoresis and its growth was tracked until acute challenge with 10^5 CFU of VPI10463 spores at 19d post-UBA3401 administration.

UBA feces extraction and *in vitro* VPI10463 growth assay

A sample of 2G of feces from UBA3401 consortium mice was homogenized in 600 µl PBS via 1 min bead beating. A 1 ml of chloroform was added to the sample and a subsequent 5-minute bead beating step was used to promote diffusion of hydrophobic compounds into the chloroform layer. Both aqueous and chloroform layers were subsequently passed through a 100um cell strainer, were aliquoted into three tubes for each type of extraction and were subjected to vacufuge (Eppendorf Concentrator) treatment at 30°C for 4 hours under high vapor pressure mode. Chloroform extracts were checked to ensure chloroform had completely evaporated leaving a solid precipitate which was dissolved in 1 ml PBS via 1 minute bead beating. Samples were passed through a 0.22 um syringe filter. Identical methods were utilized to create aqueous and chloroform extract ASF control samples.

Aliquoted extracts were combined with 1.5 ml BHIS broth to create replicate groups of aqueous and chloroform UBA or ASF extracts. Suppression of VPI10463 was assayed via inoculating each extract-BHIS sample with 10 µl of broth containing early log phase VPI prepared 24 hours prior by inoculation with 10 µl log phase VPI10463 broth culture. All steps were carried out in an anaerobic chamber under standard anaerobic conditions. Immediately following inoculation of extract-BHIS tubes 250 µl of sample was collected and DNA was extracted. Additional collections were carried out in 24-hour intervals and VPI10463 was quantified via qPCR.

16S sequencing

DNA was extracted and purified from the frozen stool and sputum samples using DNeasy 96 PowerSoil Pro QIAcube HT kit, supplemented with PowerBead Pro Plates (Qiagen). The V3-V4 region of 16S

rRNA genes were amplified using the following primers: 341F 5′TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′; 805R 5′GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′. PCR products of each sample were purified using Ampure XP magnetic purification beads. An index PCR was performed to attach dual barcodes and Illumina sequencing adapters using Nextera XT Index kit (Illumina). Final PCR products were verified on 1.5% DNA agarose gel, purified again using Ampure XP magnetic purification beads and quantified using Pico dsDNA assay (Invitrogen). An equal molar of each sample was then combined as the library. The library was diluted and spiked with 5% PhiX control (Illumina) and sequenced by Illumina MiSeq Sequencing System (2 x 250bp), and demultiplexed via the Illumina software. Due to quality issues with read 1, only read 2 was used for the 16S amplicon analysis.

Metagenomic sequencing

Samples, apart from the hybrid assembly samples, were processed (sample preparation and bioinformatic processing) as described previously (Youngblut et al., 2020). Briefly, 1 ng of DNA, extracted with the boiling lysis approach described above, was used for Nextera Tn5 tagmentation, followed by a 10 cycle PCR reaction to add barcodes and cleanup with Mag-Bind TotalPure NGS beads (Omega Biotech, Norcross, USA). Fragments of 400 to 700 base pairs were selected for with BluePippin, followed by sequencing on an Illumina HiSeq3000 instrument with 2x150 paired-end sequencing. Two samples, an ASF and ASF + UBA3401 consortium from mouse ceca, were used for hybrid assembly (Illumina short read and PacBio reads). These samples were collected from mice and stored at room temperature for 7 days with the Norgen Biotek Corporation Stool Nucleic Acid Collection and Preservation Tubes (catalog number 45660) during transport, and then at -20 until processing. DNA was extracted using the ZymoBIOMICS DNA MiniPrep Kit (Catalog # D4300) with a 10-minute bead beating step to minimize DNA shearing. DNA from this was used for both short-read sequencing as above but with an Illumina

NextSeq sequencing system, and long reads were sequenced from these samples with the Sequel II (Pacific Biosciences).

Trimming and QC of Illumina short reads, taxonomic assignments, and metagenome assemblies were carried out as described by Youngblut et al (Youngblut et al., 2020) except as noted in the following text for software versions, parameters, and reference databases. Taxonomic assignments and estimated abundances were calculated with Kraken2 and Bracken 2.2, using the Genome Taxonomy Database version 202 (Parks et al., 2020) as the reference database. PacBio data were demultiplexed and processed using the Lima software (version 2.7.1) with default settings. Demultiplexed PacBio and NextSeq short read data were used as input for Spades 3.15.4 (Prjibelski et al., 2020), using the –PacBio and --k 21,33,55,77 parameters to generate hybrid assemblies. Antimicrobials were detected with AntiSMASH version 6.0.1 (Blin et al., 2019).

Data availability statement

Sequencing data were deposited to the European Nucleotide Archive under accession PRJEB73455 (16S and Illumina HiSeq metagenomic reads) and accession PRJEB73515 (NextSeq and PacBio data for hybrid MAG assembly). All other data utilized is within the manuscript itself.

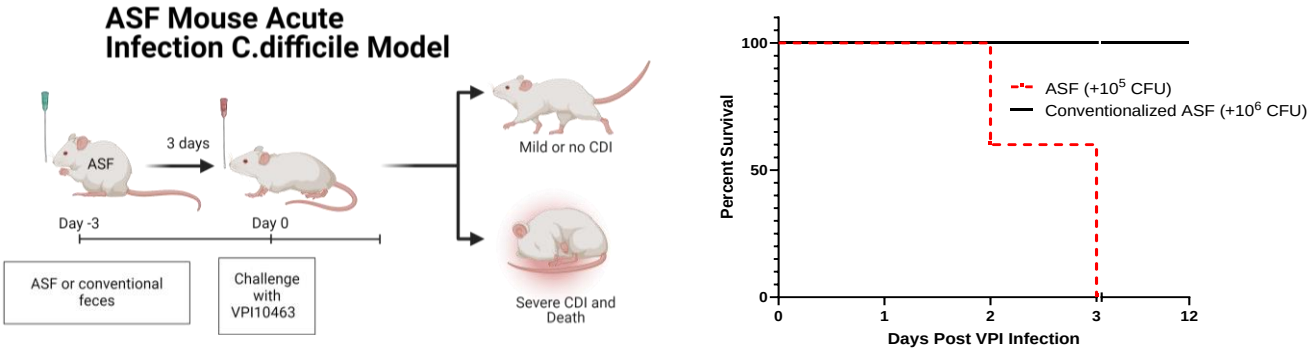
Results

ASF Mice are Prone to Acute and Chronic *C. difficile* Infection

The vast majority of mammalian encounters with *C. difficile* do not result in high levels of colonization or disease, largely reflecting the ability of the commensal gut microbiota to exclude this pathogen(Furuya-Kanamori et al., 2015). Yet, which specific bacteria contribute to such colonization resistance and how they do so is not well defined. Germfree (GF) mice are one possible platform to study how specific bacteria interact with *C. difficile in vivo* but a detrimental aspect of this approach is that GF mice have a range of abnormalities in metabolism and immune

system development. Hence, I investigated if mice with a very limited microbiota, namely Altered Schaedler Flora (ASF) mice, which lack the immune and metabolic defects of GF mice (Wymore Brand et al., 2015), might be prone to *C. difficile* colonization without use of antibiotics. I observed that, like GF mice, ASF mice were exquisitely sensitive to *C. difficile* exposure, uniformly exhibiting mortality following inoculation with 10^5 spores of *C. difficile* VPI 10463, a commonly used dose for antibiotic-treated mice **(Figure 1A)**. Administering ASF mice a complex microbiota, specifically a fecal suspension generated from conventional mice, resulted in the stark colonization resistance to CDI normally exhibited by conventional mice. Specifically, administering conventionalized ASF mice a 10-fold-higher dose (10^6 spores) of *C. difficile* VPI 10463 did not result in colonization (fecal *C. difficile* was undetectable by PCR 2-4 days post-inoculation), symptomatic disease, or mortality **(Figure 1A)** thus indicating that strong colonization resistance had been provided by the transplanted conventional microbiota.

Figure 1A



ASF mice mostly survived (75%) a lower dose *C. difficile* challenge, namely 100 spores per mouse, but were lastingly infected as indicated by high levels of *C. difficile* in their feces **(Figure 1B)**. In contrast, and in accord with numerous published studies, conventional mice administered a much higher dose of *C. difficile*, namely 10^6 spores, only barely and transiently

displayed detectable levels of fecal *C. difficile*. Such chronic *C. difficile* infection was associated with mice having an overtly ill appearance exhibiting hunched posture and low activity (these symptoms exhibited all 9 ASF mice and 0 of 5 conventional mice in Figure 1B) although histopathologic analysis (Figure 1C) did not indicate overt gut inflammation utilized (a pathologist examined tissue from 5 ASF mice with chronic symptomatic CDI but did not identify any features of overt inflammation reminiscent of CDI)**.** Attainment of such a chronic symptomatic *C. difficile* infection was also possible in GF mice but most (between 80-100% across multiple experiments) GF mice succumbed to this low-dose *C. difficile* challenge. Thus, I reasoned that ASF mice were not only a more physiologic model but also a more practical model to study how specific bacterial taxa might impede *C. difficile in vivo*.

Figure 1B

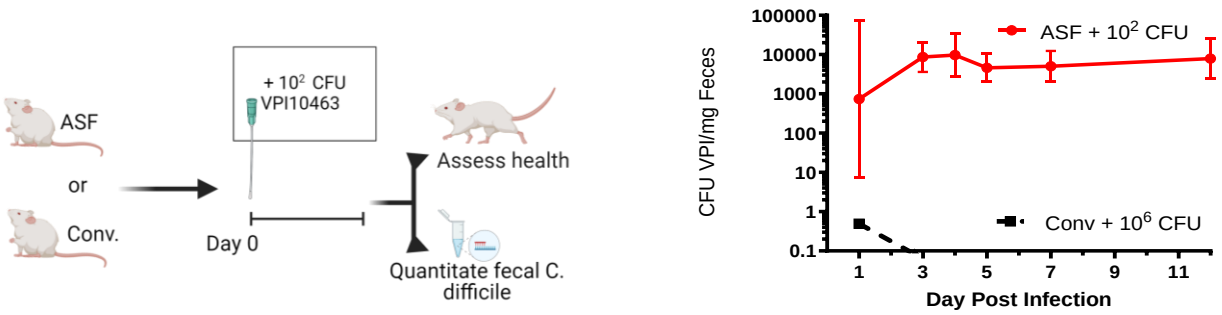


Figure 1C

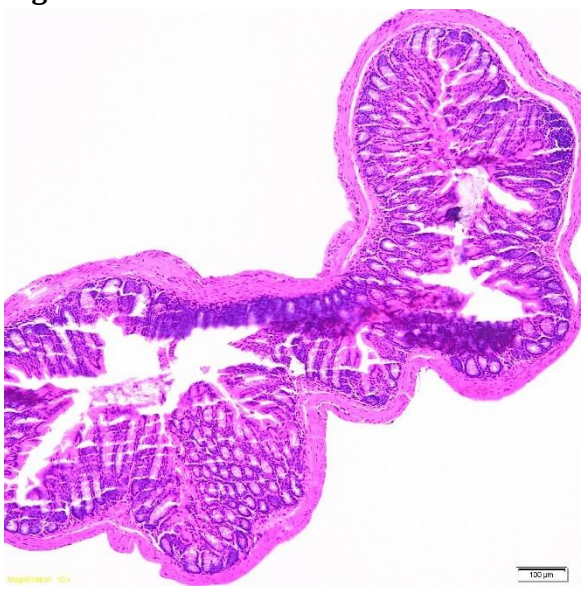


Figure 1: Altered Schaedler flora (ASF) mice were prone to acute lethal and chronic CDI. (A) Acute CDI model. Experimental schematic and Kaplan-Meier survival plot following acute CDI. ASF mice were administered feces from conventional or ASF mice and, 3d later, inoculated with 105 or 106 CFU C. difficile VPI10463, respectively (n=10). (B) Chronic C. difficile model. Experimental schematic and CFU data. Conventional or ASF mice were inoculated with 106 or 102 CFU VPI10463. Health and fecal C. difficile levels were monitored. Data are geometric means +/- CI 95%, from a single experiment (n=9 for ASF group and 5 for conventional) and representative of several experiments. (C) Proximal colon H&E of chronic CDI mice 5 weeks post infection.

Clostridia Species Provide ASF Mice Colonization Resistance to *C. difficile*

I next sought to investigate the potential ability of select classes of commensal bacteria to impact acute and/or chronic *C. difficile* infection in ASF mice. Specifically, I examined the impact of a previously described crude preparation of Clostridia **(Figure 2S)**, which had been isolated from conventional mice(Kim et al., 2017). This clostridia prep contains hundreds of species from several families including in order of relative abundance; Lachnospiraceae (32%), Ruminococcaceae (29%), Bacillaceae (7%), Enterobacteriaceae (4%), Enterococcaceae (3%) Eubacteriaceae (3%) and unclassified (13%). Administration of the clostridia prep confers colonization resistance to gram-negative facultative aerobic pathogens *S. typhimurium* and *C. rodentium* but which taxa mediated the protection was not determined (Kim et al., 2017). I herein observed that this clostridia prep also protected ASF mice against acute *C. difficile* challenge.

Specifically, administration of the Clostridia prep to ASF mice 7 days prior to inoculation with 10^5 *C. difficile* spores reduced fecal levels of *C. difficile* by over 4-logs and conferred 100% survival whereas all untreated ASF mice died within 3d of *C. difficile* administration precluding sample collection past the second day **(Figure 2A)**.

Figure 2A

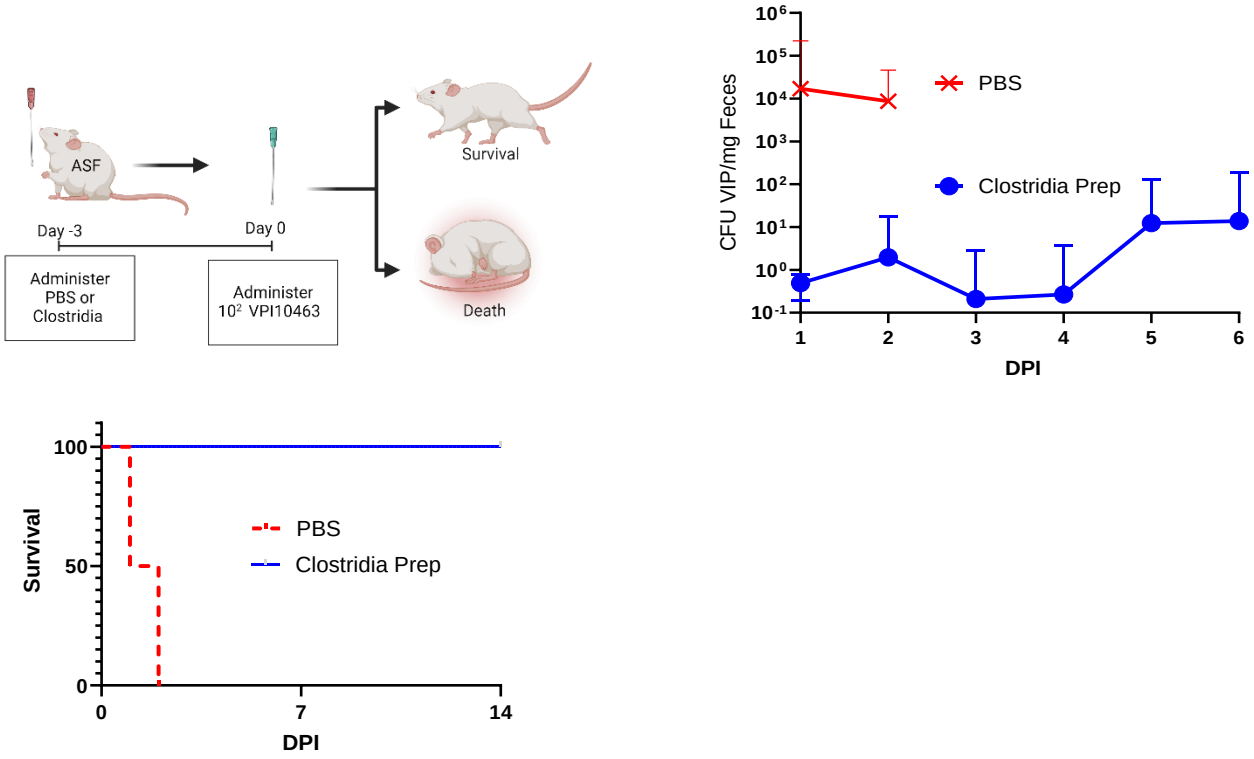


Figure 2: A Clostridia Consortium protected ASF mice against acute and chronic CDI. (A) ASF Mice were administered PBS or Clostridia prep 3 days prior to challenge with 10^4CFU VPI10463. Levels of fecal VPI and survival were monitored on indicated day post inoculation. Data are means +/- S.E.M. (n=4, 2M/2F for PBS group and 5 2M/3F for Clostridia group).

I next administered the clostridia prep to ASF mice with established chronic *C. difficile* infection, achieved via a low-dose inoculation 21d prior. Chronically infected mice that received the Clostridia prep displayed a stark drop in *C. difficile* levels within a week, and thereafter displayed only barely detectable levels of this pathogen **(Figure 2B)**. In contrast, mice

administered vehicle (PBS) continued to display high levels of *C. difficile* in their feces. Reductions in *C. difficile* levels following administration of the Clostridia prep was associated with restoration of indices of health readily evident by a near complete elimination of visible disease symptoms. These results indicated that use of ASF mice to model acute and chronic *C. difficile* infection could serve to investigate microbiota-mediated amelioration of this condition.

Figure 2B

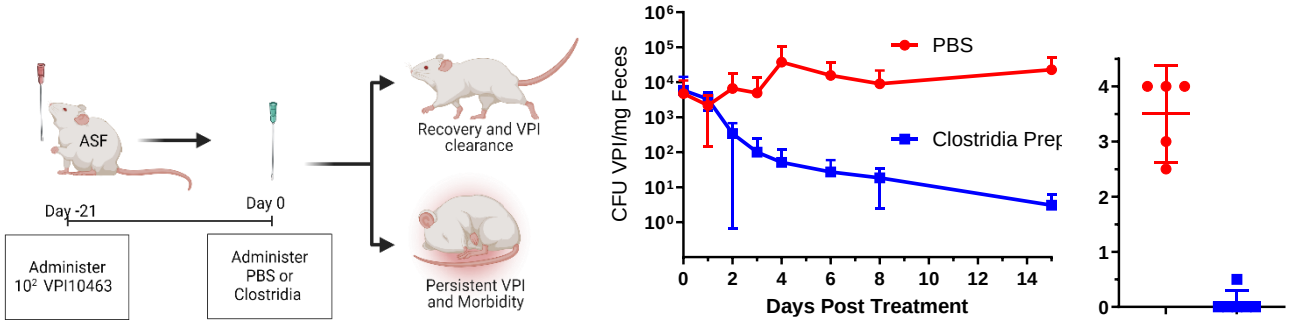


Figure 2: (B) ASF mice were inoculated with 10^2 CFU of VPI10463 to establish a chronic infection. 21 d later, mice were administered PBS or Clostridia prep by oral gavage (referred to as d0 post-treatment). Fecal CFU was monitored on indicated day post-treatment. 14 days later, mice were assessed for dehydration, grimace, mobility, and rectal inflammation, by a scientist blinded to study conditions, who gave a symptom severity score of 0-5 with 0 corresponding to asymptomatic and 5 severe CDI. Data are means +/- S.E.M. (n=5, 2M/3F for PBS and 6, 3M/3F, for Clostridia prep).
(n=4, 2M/2F for PBS group and 5 2M/3F for Clostridia group).

Figure 2S

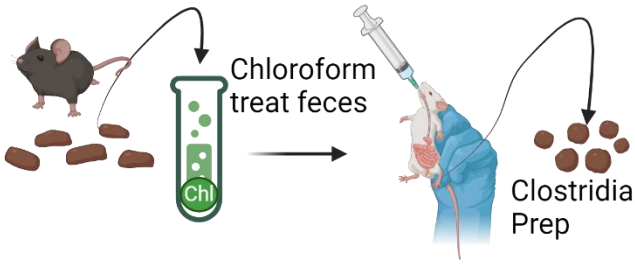


Figure 2: (S) generation of clostridia prep by treating conventionally housed adult mouse feces with 100% chloroform to eliminate all non-spore forming microbes.

Protection Against *C. difficile* Associated with *Lachnospiraceae*

I presumed that the protection against CDI by the Clostridia prep reflected such microbes and/or their products, acting directly upon *C. difficile*. Yet it also seemed conceivable that

activation of the immune system might be involved. Hence, to investigate whether the adaptive arm of the immune system played a role, I used GF Rag1KO mice, which are broadly deficient in adaptive immunity. As expected, all mice in the control (PBS) group mice succumbed to *C. difficile* by 48 hours post-inoculation. In contrast all GF Rag1KO mice that received the clostridia prep survived **(Figure 3B)** thus arguing against a role for adaptive immunity in mediating the Clostridia prep's protection against CDI. I next turned our attention to discerning the key components of the Clostridia prep that mediated its ability to protect against *C. difficile.* The Clostridia prep is a chloroform generated derivative of conventional mouse feces and thought to be comprised of hundreds of microbial species with members of the class Clostridia predominating[14]. The Clostridia prep itself is maintained by administering it to GF mice and then isolating their feces. In an attempt to capture the protective microbes in cell culture, such fecal pellets were anaerobically incubated in BHIS for 3d and then plated on BHIS agar. All colonies were collected, pooled, and replated. Such collection of pooled colonies on the second plate was referred to as the cultured Clostridia prep (**Figure 3A**). This cultured-Clostridia prep also protected GF Rag1KO mice against *C. difficile*-induced mortality although fecal *C. difficile* levels its ability to impede the pathogen was much less than the starting Clostridia prep **(Figure 3B)**. I also generated a heated version of the Clostridia prep by incubating a fecal suspension of it to 55^0C for 30 minutes prior to initially plating it on BHIS agar. Such heated Clostridia prep provided against acute CDI at least as well as the original clostridia prep. Collectively, these results suggested that the key protective microbe(s) had limited capacity to grow under standard anaerobic culture conditions but were capable of generating heat-stable spores that could germinate in the mouse intestine.

Figure 3A

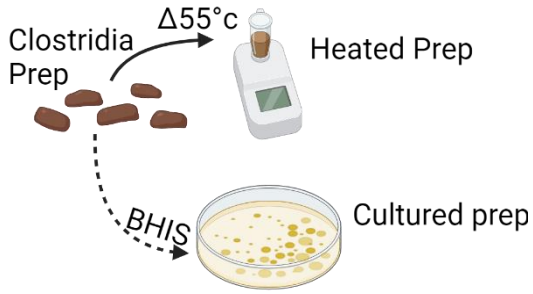


Figure 3B

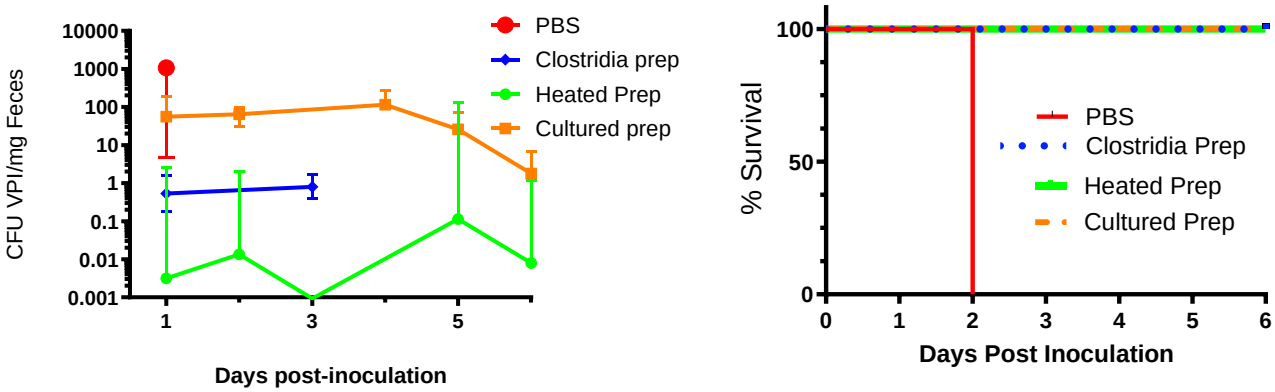


Figure 3: Clostridia prep's protection against CDI is independent of host immunity and associates with *Lachnospiraceae*. (A) Schematic of how cultured clostridia prep and heated clostridia prep were generated. **(B)** Germ-Free Rag1KO mice were administered the Clostridia prep or fractionated version thereof as indicated. 7d later, mice were inoculated with 10^3 spores of VPI10463. Survival and fecal *C. difficile* levels were monitored. Data are means +/- S.E.M. (n=4). Some groups of mice failed to produce fecal samples on some individual days.

I next administered the original Clostridia prep, the heated Clostridia prep and the cultured Clostridia prep to ASF mice with established chronic *C. difficile* infections. Analogous to results in the acute model, the cultured Clostridia prep had modest impacts on *C. difficile* levels while the original Clostridia preparation and its heated derivative both starkly lowered *C. difficile* levels within 7d of their administration **(Figure 3C)**. I next analyzed microbiotas of these mice *via* shotgun metagenomic sequencing, over time, seeking to identify taxa whose appearance associated with the disappearance of *C. difficile*. Family levels analysis revealed that, not surprisingly, *Tannerellaceae*, the family to which most ASF bacteria, including the predominant ASF microbe, *Parabacteroides goldsteinii* (ASF519), belongs was prominent in all samples. As further expected, *Peptostreptococcaceae*, the family to which *C. difficile* belongs, was also

apparent in these chronically infected mice. More interestingly, I also observed the appearance of *Lachnospiraceae* associating with disappearance of *Peptostreptococcaceae* in treating mice resolving the infection. More specifically, Lachnospiraceae was a predominant family (43-76 % of reads) in mice receiving that had cleared C. difficile but was a relatively minor component of the microbiota (under 22%) in the mice that had not. This inverse correlation between *C. difficile* and *Lachnospiraceae* was appreciable by plotting the relative amounts of *Lachnospiraceae* and *C. difficile* in individual samples**.** This led us to hypothesize that *Lachnospiraceae* species might provide colonization resistance against *C. difficile*.

Figure 3C

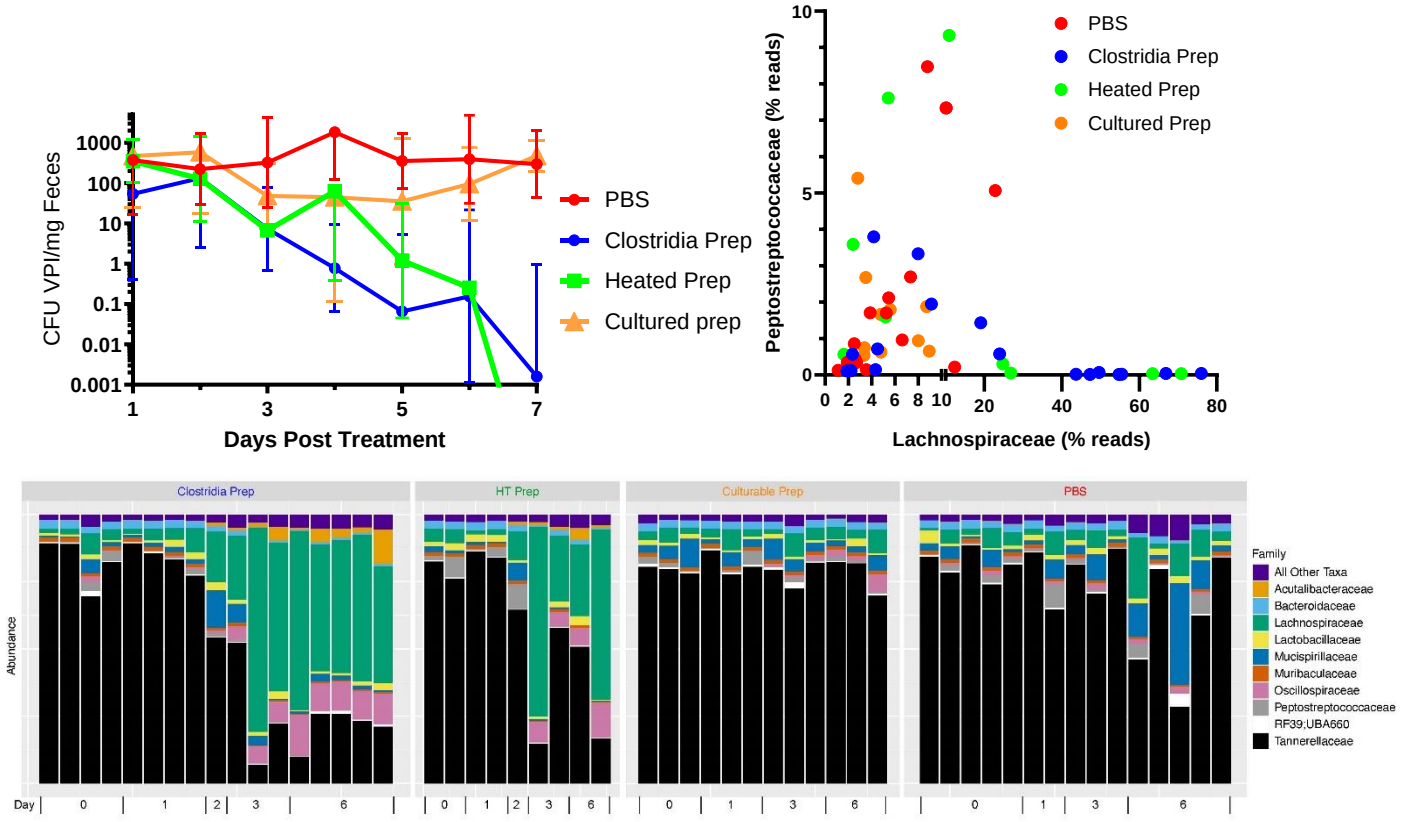


Figure 3S

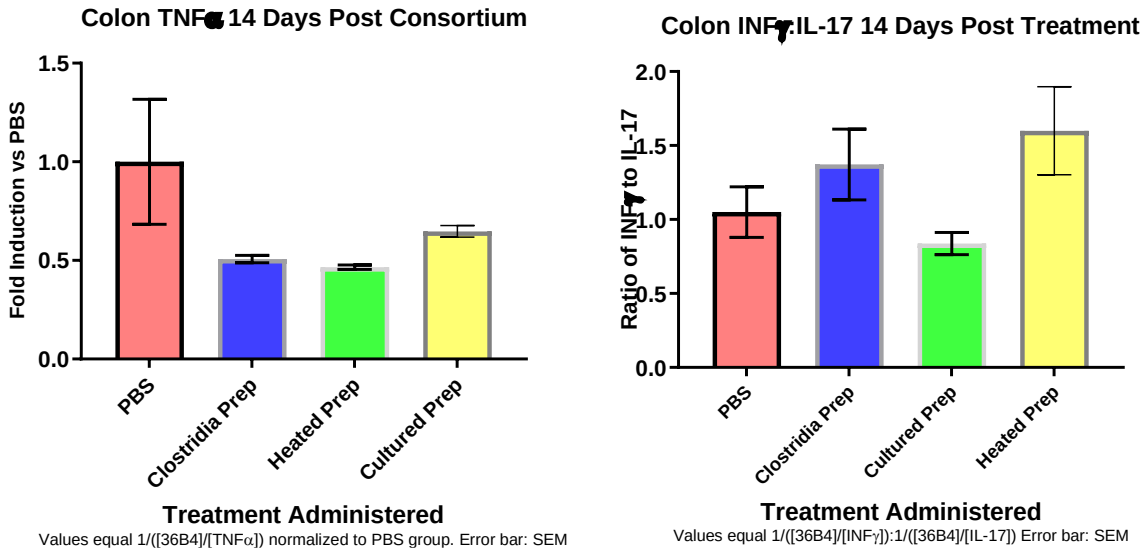


Figure 3: (C) ASF mice with established chronic CDI (as result of inoculation with 102 CFU of VPI10463 21d prior) were administered the Clostridia prep, or derivative thereof, as indicated. Fecal samples were subjected to qPCR to measure C. difficile levels. Data are means +/- S.E.M. (n=3-5). Select samples were also subjected to shotgun metagenomic sequencing. Bar chart shows taxonomy at family level at indicated time point following administration of indicated treatment. Point plot shows percentage of reads assigned to Lachnospiraceae or Peptostreptococcaceae (family to which C. difficile belongs) for individual samples from each condition. **(S)** cytokines from same experiment at 14 days post treatment, colon TNFα mRNA fold induction relative to PBS, and the INF-γ:IL-17 cytokine ratio.

Although the small sample sizes precluded statistical significance, measurement of cytokine transcripts demonstrated reduced markers of acute inflammation in mice that had received the Clostridia preparations (**Figure 3S**). Additionally, the ratio of INF-γ to IL-17, found from clinical use to hold an inverse correlation with CDI severity was elevated in mice that received the Clostridia preparations, along with improved inflammatory markers suggesting that treatment mice were recovering to a healthier state. (Yu et al., 2017)

Figure 4A

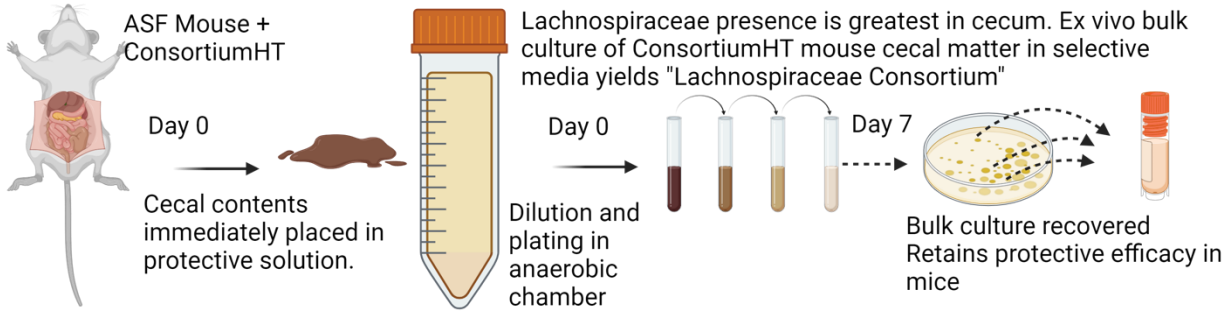


Figure 4. Protection against CDI by a *Lachnospiraceae*-enriched consortium associates with levels of *Lachnospiraceae* UBA3401. (A) Schematic illustration of approach used to generate a *Lachnospiraceae*-enriched consortium from the Clostridia prep.

Lachnospiraceae-mediated ProtectionAagainst *C. difficile* Associates with UBA3401

I reasoned that, although our ability to propagate the *C. difficile* suppressing microbes *ex vivo* was quite limited, it nonetheless might provide an opportunity to investigate our hypothesis that *Lachnospiraceae* had suppressed *C. difficile* in our model. I isolated cecal contents from ASF mice that had been administered the heat-treated singly passaged Clostridia prep and sought to culture it using conditions known to favor *Lachnospiraceae*. Accordingly, testing of an array of individual selected colonies by 16S sequencing confirmed predominance of *Lachnospiraceae* family members. I then pooled the colonies collected from individual plates, generating glycerol stocks that were stored at -20C, and hereafter referred to as a *Lachnospiraceae*-enriched consortia (**Figure 4A**). The inherent resistance of obligate anaerobes to aminoglycosides, (Bryan & Kwan, 1981) and susceptibility of gram negative microbes to aztreonam were used to create a media selective for *Lachnospiraceae* (**Figure 4S**). Analogous to the original Clostridia preparation, the *Lachnospiraceae*-enriched consortia provided ASF mice stark protection against high-dose *C. difficile* challenge (**Figure 4B**) and, moreover, starkly reduced *C. difficile* levels in ASF mice with previously established chronic *C. difficile* infections (**Figure 4C**). To identify specific *Lachnospiraceae* species that had potentially helped clear *C. difficile*, feces from these mice were subjected to shotgun metagenomic sequencing, hoping the reduced complexity

relative to the previous samples might aid species level analysis. Indeed, species level taxonomy bar charts revealed the reduction in *C. difficile* in mice receiving the consortia associated with appearance of a *Lachnospiraceae* species, namely Uncultured Bacteria and Archaea (UBA) 3401 (ANI is ~99.4%, as calculated by FastANI is ~99.4%), a bacterium, which, as its name implies, is only known to exist as one of the thousands of intestinal bacterial genomes assembled from existing databases of shotgun sequencing (Parks et al., 2017). The inverse association of UBA3401 and *C. difficile* was most readily appreciated via a point plot of the relative abundance (% reads) of these species in individual samples. Hence, I hypothesized that UBA3401 might have a strong ability to impede *C. difficile*.

Figure 4B

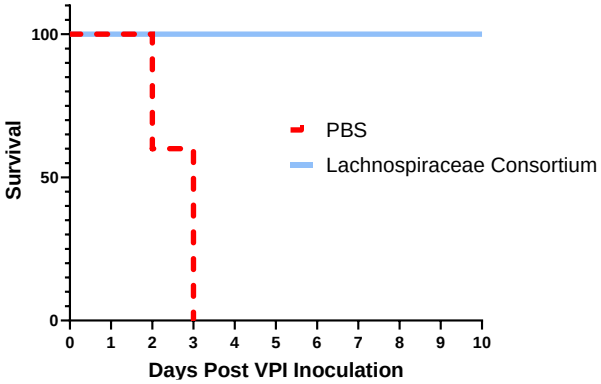


Figure 4C

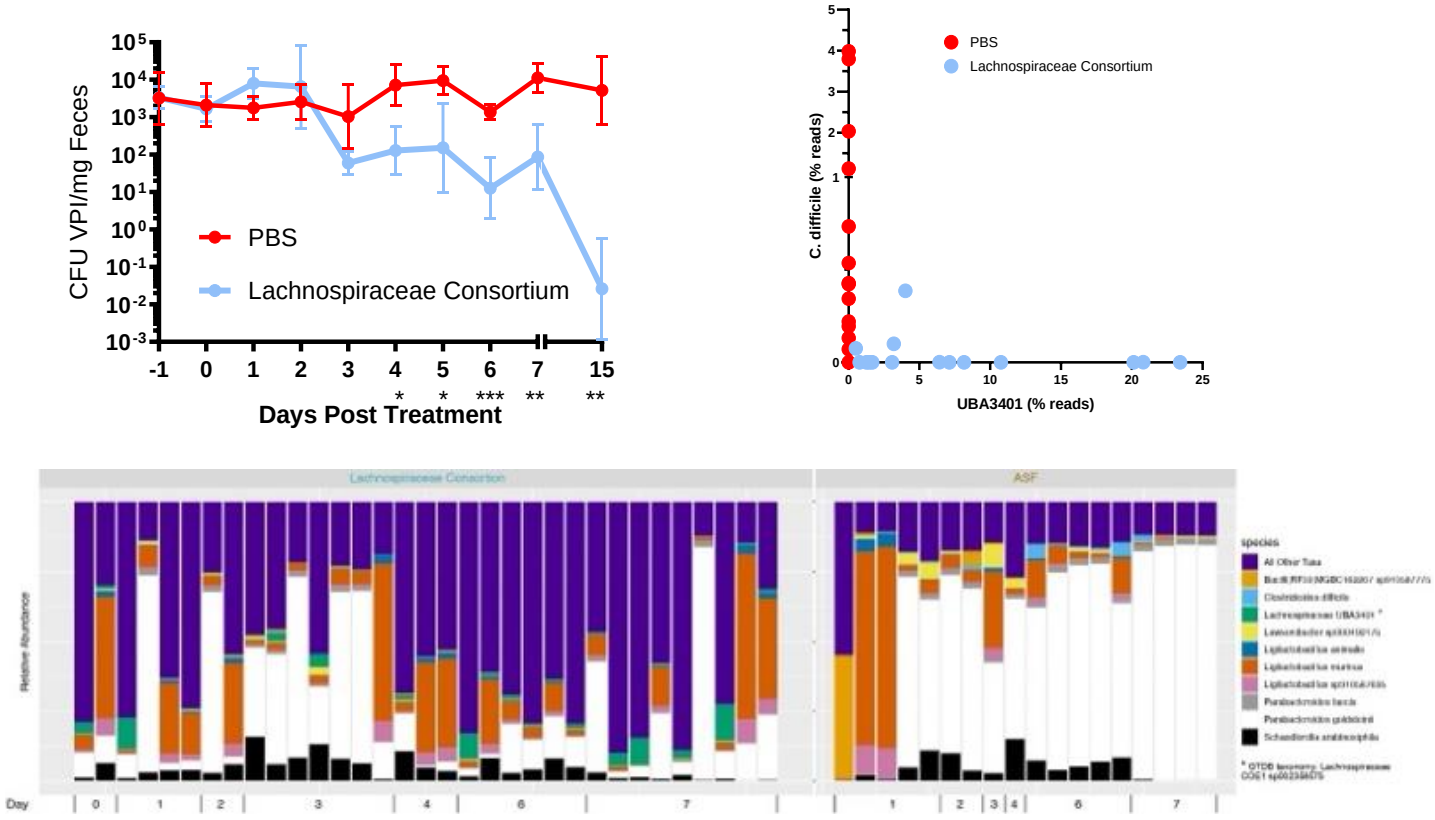


Figure 4S

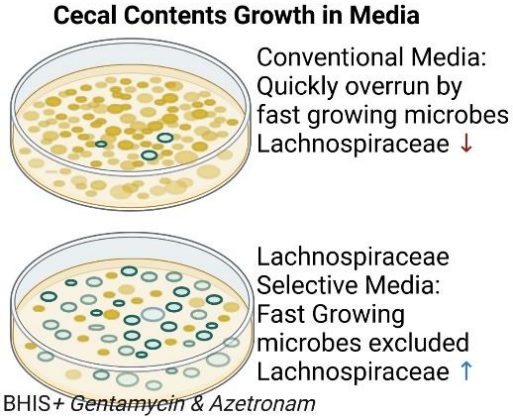


Figure 4. (B) ASF mice were administered PBS or *Lachnospiraceae*-enriched consortium. 7d later, were challenged with 10^5 VPI10463 (n=5). **(C)** ASF mice with established chronic CDI (as result of inoculation with 10^2 CFU of VPI10463 21d prior) were administered PBS or *Lachnospiraceae*-enriched consortium. Fecal samples were subjected to qPCR to measure *C. difficile* levels. Data are means +/- S.E.M. (n=6, 3M/3F). Select samples were also subjected to shotgun metagenomic sequencing. Bar chart shows species level taxonomy at indicated time point following administration of indicated treatment. Point plot shows percentage of reads assigned to *C. difficile* and UBA3401 for individual samples from each condition. **(S)** Improved cultivation of *Lachnospiraceae* following use of selective media.

Generation of ASF/UBA3401-Colonized Mice

The previously reported partial UBA3401 genome (NCBI: txid1952033), albeit incomplete, was used to design primers capable of specifically recognizing the presence of this bacteria. I found those corresponding to bases between 2241 and 2669 of the publicly available GCA_002358575.1 UBA3401 genome to be distinct from other microbes including those of the *Lachnospiraceae* family (**Figure 5A**). Primers targeting this region successfully amplified the expected 429 BP fragment from fecal samples of ASF mice that had received the *Lachnospiraceae*-enriched consortium (**Figure 5B**). Such a PCR product was not observed when using feces from conventional or conventionalized mice although quantitative (q) PCR found amplification at high cycle numbers, suggesting UBA3401 is present at low levels in non-manipulated microbiomes and that our gel-based assay has a higher threshold of UBA3401 detection. As expected, those samples showing the presumed UBA3401-specific PCR product on gels displayed qPCR positivity at relatively low Ct numbers, which, when compared to Ct values obtained with universal 16S primers, served to identify the samples with greatest relative abundance of UBA3401 **(Figure 5B).** I next used these PCR assays to monitor attempted *in vitro* cultivation of UBA3401. Feces and cecal contents, suspended in deoxygenated PBS, were cultured, anaerobically, under a wide variety of conditions, and numerous colonies screened for presence of UBA3401. In contrast to most conditions, which failed to yield any positive colonies, culture on BHIS enriched with defibrinated sheep blood, taurocholate, and cellobiose produced several positive colonies. However, subculture of these colonies produced colonies of similar appearance but yet no longer PCR-positive for UBA3401, suggesting that growth of this bacterium may require a syntrophic partner.

Figure 5A

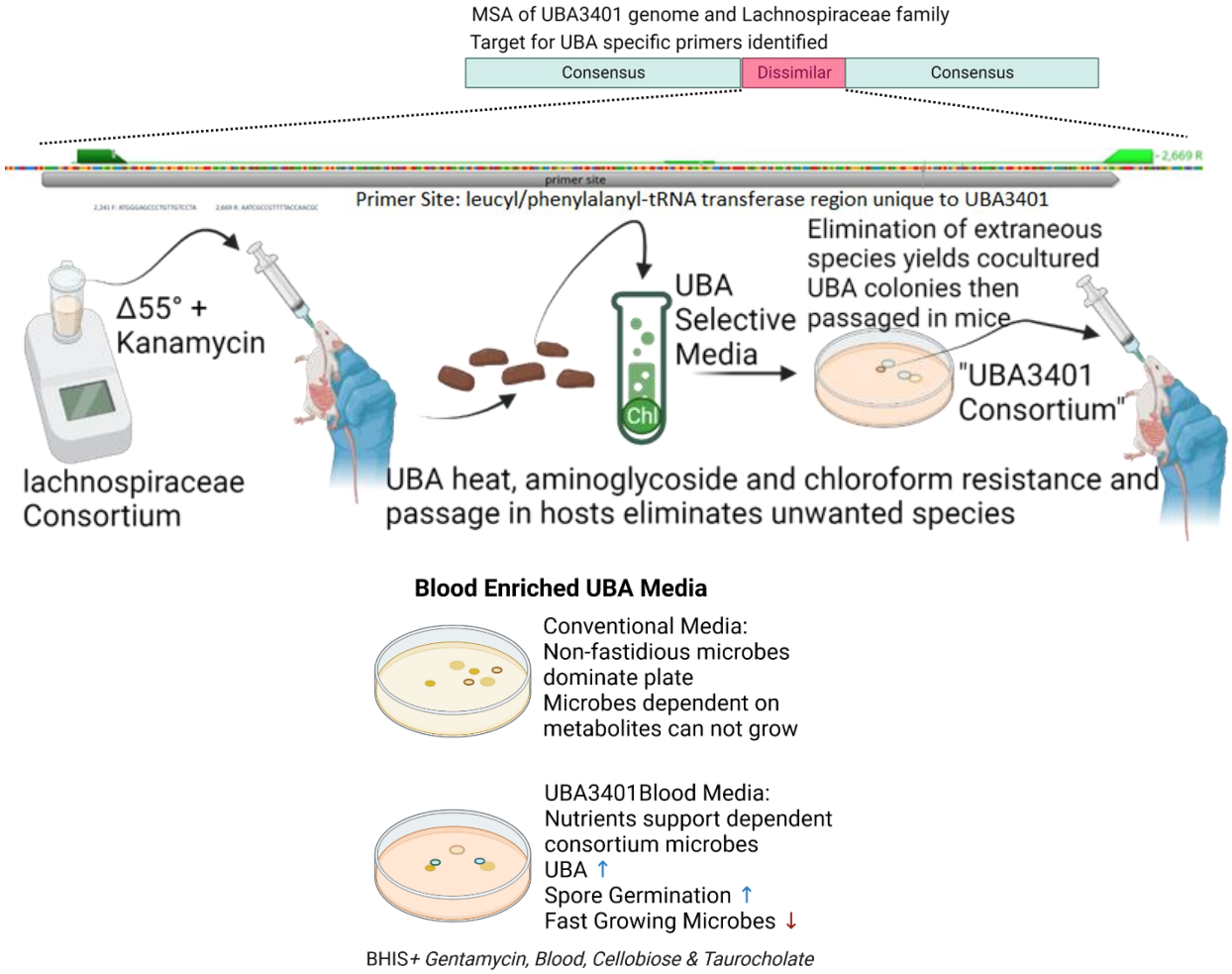


Figure 5. Creation of UBA3401-enriched consortium in ASF mice. (A) Generation of UBA3401 specific primers targeting a unique region in the databank UBA3401 genome and methods for creation of UBA3401 consortium.

Figure 5 B

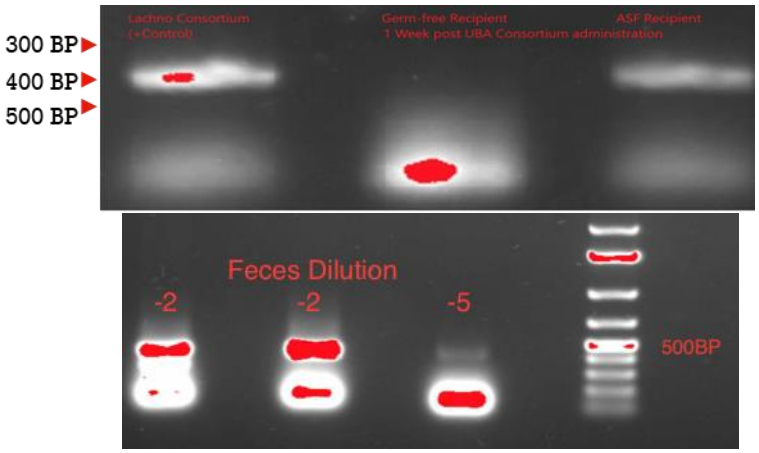
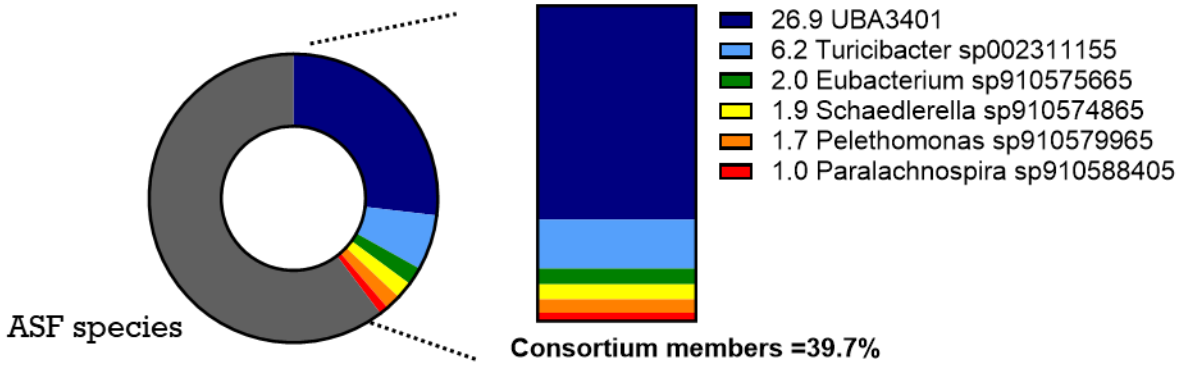


Figure 5 (B) (**Top**) Gel-based detection of UBA3401 using primers generated in A. Sample lanes were: 1)Lachno-enriched consortia. 2) feces from germ-free mice used in limiting dilution transplants UBA3401 that qPCR suggested may be monoassociated with UBA3401 at low abundance. 3) ASF mouse administered fecal sample from lane 2; (**Bottom**) feces from ASF mice 7 days following administration of 10^2 and 10^5 fold diluted UBA3401 consortium. 429BP bands differs in intensity and qPCR detection threshold.

In parallel, I sought to isolate UBA3401 *in vivo* by limiting dilution fecal transplant into germfree mice, followed by selecting mice with the highest relative abundance of UBA3401 based on Ct values using UBA-specific and universal 16S primers. Multiple rounds of this approach led to the generation of mice that were potentially mono associated with UBA3401 based on Ct values and that 16S sequencing yielded results consistent with mice being mono associated with UBA3401 but the absolute amount of bacterial DNA in fecal samples from such mice was very low. These *in vivo* results further supported the suggestion that UBA3401 is highly recalcitrant to growing without the presence of other commensal members. However, transfer of these feces harboring UBA3401 at low abundance to ASF mice led to an approximately 30-fold higher absolute abundance of UBA3401 and, concomitantly, a readily observable band on a gel. Sequence analysis indicated that such mice were indeed enriched in *Lachnospiraceae*, particularly UBA3401 (**Figure 5C**). With UBA3401 growing to become the

predominant non ASF microbe within 2 weeks (**figure 5S**) Consequently, I used ASF mice as a means of propagating UBA3401.

Figure 5C



Name	Estimated Abundance
Parabacteroides goldsteinii	25.48%
Lachnospiraceae bacterium UBA3401	20.61%
Ligilactobacillus murinus	16.56%
Turicibacter sp002311155	10.24%
Lachnospiraceae bacterium UBA3404	6.93%
Mucispirillum schaedleri	4.39%
Eubacterium MGBC164771	3.49%
Lawsonibacter MGBC000555	2.35%
Schaedlerella MGBC163752	1.94%
Clostridium sp. MD294	1.54%
(Remaining)	6.46%

Figure 5S

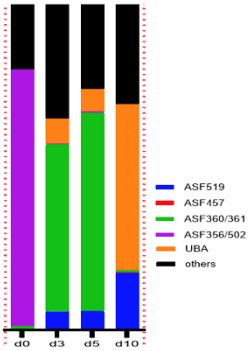


Figure 5. (C) (**Top**) Metagenomic sequencing analysis of sample from lane 3. Results indicated that UBA3401 was the predominant non-ASF taxa in "UBA3401-enriched" ASF mice. (**Botom**) Corroborating sequencing analysis of identical sample carried out by Transnetyx. **(S)** 16s sequencing of ASF mouse fecal pellets from days 0 to 10 following administration of UBA3401 consortium.

UBA3401 Genome and Potential Mechanisms of Colonization Resistance

Feces from UBA3401-colonized ASF mice were further analyzed by in-depth shotgun and long-read metagenomic sequencing. Sequences were assembled to generate a UBA3401 genome estimated to approach 100% completion with 4.11% contamination. The genome appeared broadly similar to other *Lachnospiraceae;* however, analysis of the genome revealed the presence of a region closely homologous to known thiopeptide antibiotic genes **(Figure 6A)**. The putative thiopeptide possessed organization consistent with known thiopeptide biosynthetic gene clusters (BGC) including in it a YcaO enzyme at the end of the BGC.(Vinogradov & Suga, 2020) I also noted an ABC transporter and efflux transporter located immediately downstream of the predicted thiopeptide BGC. Additionally, multiple genes implicated in regulation of biosynthesis such as cyclic lactone autoinducers, along with quorum sensing ranthipeptide genes (Chen et al., 2020; Mull et al., 2018) were found in the UBA3401 genome. Furthermore, a species that was also present in the UBA3401/ASF feces, namely *Paralachnospira sp910588405,* was found to have potential genes predicted to code for betalactone antibiotic production and ranthipeptide quorum sensing. These genomic elements suggest that UBA3401 might release products with direct anti *C. difficile* action. To test this notion, I mixed early log phase *C. difficile* cultures with extracts of feces from ASF and UBA3401-enriched ASF mice and monitored *C. difficile* growth. I found that, relative to ASF fecal extracts, addition of fecal extracts from UBA3401/ASF mice reduced *C. difficile* growth *in vitro*, thus supporting the notion that UBA3401 may limit *C. difficile* growth *via* secreted products (**Figure 6B**).

Figure 6A

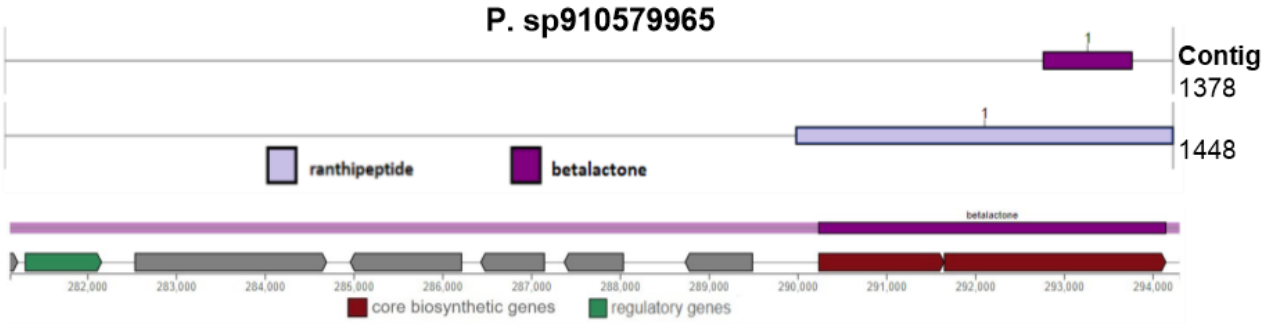


Figure 6B

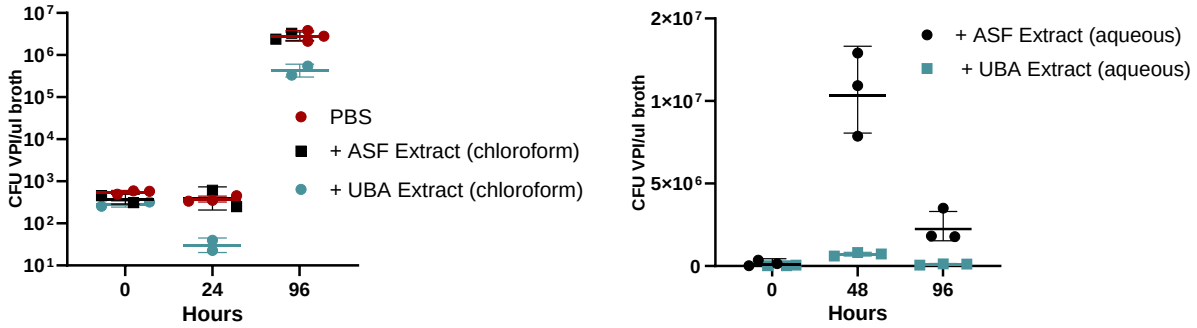


Figure 6: In silica and *in vitro* analysis indicate UBA3401 may directly impede *C. difficile* growth. (A) Analysis of the UBA3401 genome identified putative antimicrobial genes as discussed in text. **(B)** Extracts of ASF/UBA3401 colonized mice, but not ASF mice, impede *C. difficile* growth *in vitro*. Low density cultures of *C. difficile* VPI10463 were administered extracts of feces from UBA and UBA3401 mice as described in Methods. *C. difficile* levels measured by PCR at indicated time points. * Indicates significant difference between ASF and ASF/UBA3401, p, 0.05.

UBA3401 Protects Mice Against *C. difficile*

Lastly, I sought to examine the potential of UBA3401 to protect against *C. difficile* colonization and disease *in vivo*. Groups of ASF mice were administered cecal suspensions generated from ASF or UBA3401/ASF mice (**Figure 7A**). Monitoring UBA3401 engraftment by PCR found it was detectable at low levels within a few days post-administration. It then increased slowly over the course of 2 weeks at which point it plateaued. 16S rRNA gene sequence analysis of such fecal samples pre- and post-administration of the cecal transplants supported the notion that UBA3401 was the predominant, albeit not the only bacteria introduced

into the ASF microbiota **(Figure 7B)**. In particular, I noted the appearance of *Turicibacter*, frequently observed in our various attempts to isolate UBA3401 at early time points preceding growth UBA3401. 20 days post-microbiota transplant, mice were challenged with 10^5 spores of *C. difficile*. As expected, recipients of ASF microbiotas remained highly prone to CDI with all mice displaying high levels of fecal *C. difficile* 1d post-challenge. Concomitantly, such mice appeared overtly ill, lost weight, and died within 3d **(Figure 7C)**. In contrast, recipients of UBA3401-containing microbiotas displayed only modest *C. difficile* loads and lacked clinical indices of disease. Yet, analysis of their feces by sequencing and PCR found that, unlike in the above-described experiments wherein ASF mice were administered whole Clostridia preps, recipients of UBA3401-enriched microbiotas did not completely clear the pathogen. Nonetheless, collectively, these results accord with UBA3401 providing colonization resistance against *C. difficile*.

Figure 7A

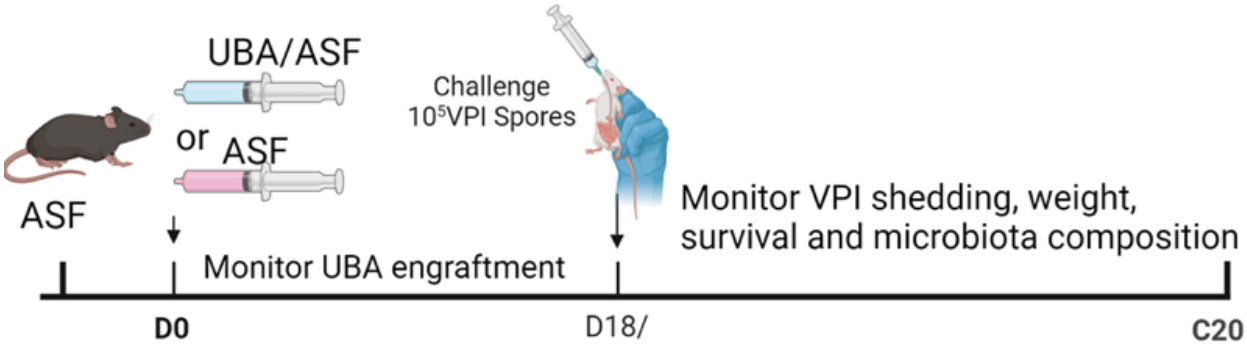


Figure 7B

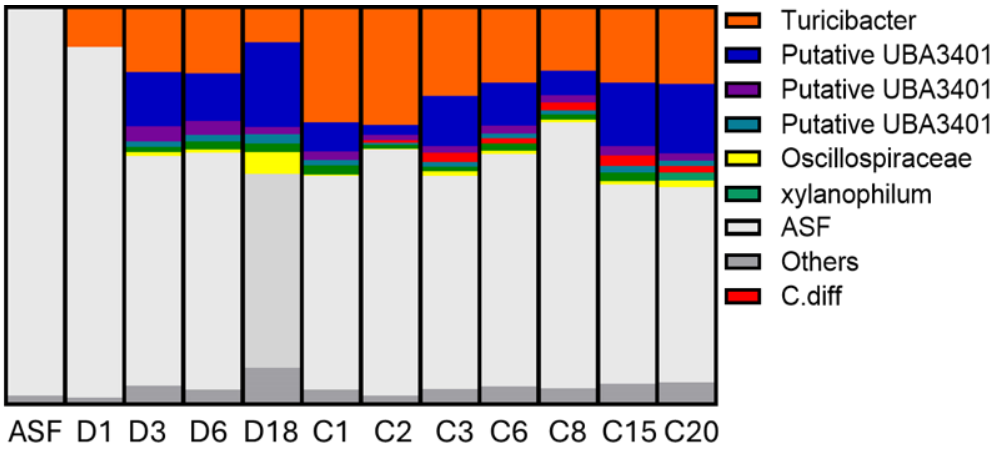


Figure 7C

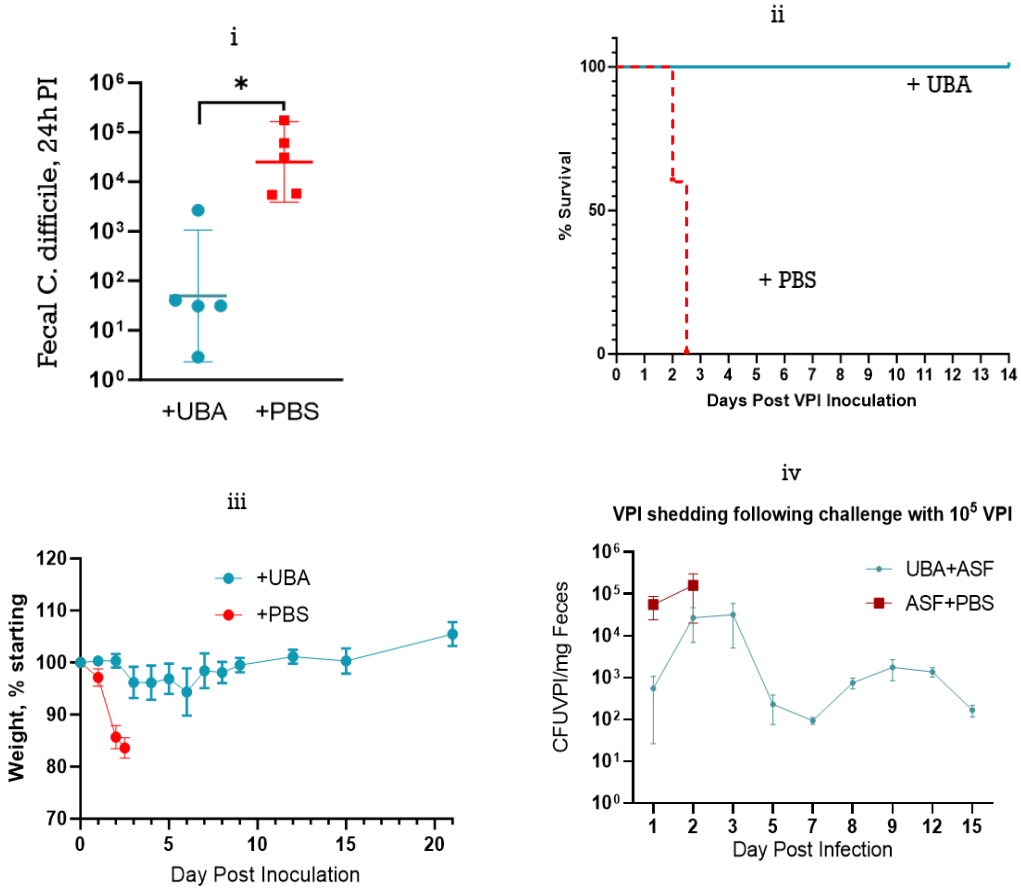


Figure 7S

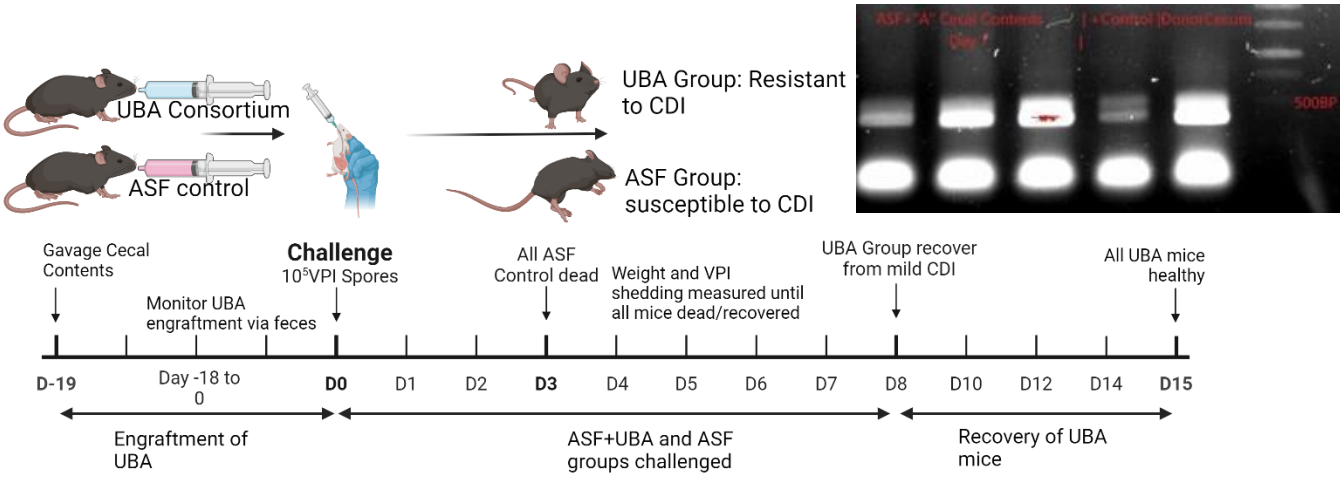


Figure 7: UBA3401 administration protects against CDI. (A) Experimental design schematic. **(B)** Microbiota analysis via 16S sequencing of feces from ASF mice on indicated day following administration of UBA3401-containing feces **(D)** and, subsequently, days following *C. difficile* administration (C). **(C)** Consequences of administering ASF3401 feces to ASF mice on *C. difficile* infection. i) *C. difficile* loads 24h following inoculation with 10^5 VPI10463. ii) Survival. iii) Body weight. iv) C. difficile loads throughout experiment with 105 VPI10463. Data are means +/- SD (n=5, 3M/2F). * Indicates statistical significance, p, 0.05. **(S)** detailed experimental timeline with key events noted, and strongly positive UBA3401 product in ASF mice at 7 days following UBA3401 consortium administration.

Discussion

C. difficile remains a major recalcitrant public health menace. Novel approaches to prevent and treat primary and recurrent infections are needed. I hypothesize that key insights into developing new strategies to combat *C. difficile* might come from better understanding of how the gut microbiota provides colonization resistance against this pathogen. Attaining such understanding has been stymied by the complexity and heterogeneity of the gut microbiota. This study sought to surmount this hurdle via use of gnotobiotic mice that were colonized with a minimal microbiota, specifically Altered Schaedler Flora (ASF) mice, which, in contrast to germfree mice, are relatively immunologically normal. I found that ASF mice were highly prone to *C. difficile* infection (CDI) and disease without use of antibiotics and, moreover, could serve as a platform to identify bacteria capable of preventing or clearing *C. difficile* infection. Specifically, I found that a complex undefined preparation of Clostridia provided stark protection

against CDI and that a significant portion of this protection could be recapitulated by a highly fractionated derivative of this, namely one enriched in *Lachnospiraceae* UBA3401. I hypothesize that understanding how UBA3401 impedes CDI might suggest novel approaches to treat this condition. Furthermore, I envisage that pure UBA3401 cultures and/or spores might, if obtainable, serve to treat and/or prevent CDI.

Some bacteria, for example segmented filamentous bacteria, that are recalcitrant to growth *in vitro* achieve high levels when mono-colonizing mice, suggesting a requirement for a host-derived metabolite (Klaasen et al., 1991). In contrast, limiting dilution transplant of UBA3401-containing feces resulted in mice seemingly mono associated with this microbe but absolute levels were extremely low, suggesting its growth might, in addition, require metabolites provided by other bacteria, thus explaining why UBA3401 thrived in ASF mice. In this scenario, such UBA3401-supporting bacteria may be ASF species and/or other bacteria on the original consortium that engrafted with UBA3401. Extensive efforts to culture UBA3401 were unsuccessful. Indeed, I designed a variety of media types seeking to promote growth of UBA3401, including use of gentamycin and Aztreonam to suppress aerobic and gram-negative microbes respectively, and the use of blood and taurocholate to facilitate and induce growth of endospores. On several occasions, colonies initially testing positive for UBA3401 were obtained but, following subculture, they had 16S sequences that indicated they were not UBA3401 but were consistent with Blautia species thus suggesting that UBA3401 might closely associate with Blautia species. On the other hand, the absence of UBA3401 in such sub-cultures indicates such *Blautia* were not sufficient to support UBA3401 growth. Supplementing growth media with extracts from ASF mice was also to no avail, suggesting that the assistance this bacterium attains

from other microbes and/or the host, may be short-lived and/or require contact or close proximity for delivery.

How UBA3401 limits *C. difficile* colonization, and consequently disease, is not yet clear but analysis of the UBA3401 genome and our experimental observations suggest this bacterium may secrete factors that directly impede *C. difficile* growth. The UBA3401 genome contains an array of cyclic lactone autoinducers, which are associated with, and are thought to regulate bacteriocin production (Aziz et al., 2022; Zhang et al., 2023). Moreover, this segment of the UBA3401 genome encodes a secreted thiopeptide predicted to have direct antibacterial activity. Indeed, the thiopeptide spectrum of activity is directed against gram positive microbes with derivatives known to have activity against C. difficile (Kim et al., 2022). In accordance with this notion, extracts of feces from UBA3401/ASF mice directly suppressed *C. difficile* growth *in vitro*. Cyclic lactone autoinducers also mediate quorum-sensing-mediated gene expression and, consequently, may explain why some aspects of UBA3401's activity are reminiscent of quorum induced phenotypes (Ji et al., 2005). In particular, following administration of UBA3401-enriched microbes into ASF mice, we've observed a considerable lag in UBA3401 growth and, moreover, in its protection against CDI. Hence, I envisage that, following its administration to mice, UBA3401 initially proliferates slowly until other, yet to be defined bacteria that congregate with UBA3401, become established. This is followed by enhancement of UBA3401 growth and protection against *C. difficile* analogous to other examples of bacterial quorum sensing amensalism, where production of bacteriocin is only induced after signaling mass within a microbial community is reached (schematized in Figure 8) (Maldonado-Barragan & West, 2020; Quadri, 2002). Indeed, induction of bacteriocin production by mixed species consortia is

widespread in nature with addition of a supportive species greatly increasing bacteriocin production (Chanos & Mygind, 2016; Maldonado et al., 2004).

Both UBA3401 growth and expression of bacteriocins appear to be dependent upon other microbes underscores the complexity of the gut microbiota and, consequently, the difficulty in seeking to precisely manipulate it. Indeed, hosts with normal microbiotas, that have not been perturbed by antibiotics, show near complete resistance to *C. difficile* colonization. Furthermore, while undefined fecal transplants are highly effective at preventing *C. difficile* recurrence, replicating such efficacy with fully defined bacterial cocktails has proven far more difficult. In this context, I conclude that UBA3401 may be one bacterium capable of strongly impeding *C. difficile*. Consequently, better understanding the growth of this bacterium, the regulation of its gene expression and mechanism by which it impedes *C. difficile* growth, may eventuate in better and safer strategies to counteract this pathogen.

Perspective

This study homed in on ASF mice as a candidate for an improved animal model of CDI. It was hoped that the defined microbiome and metagenome of the ASF flora would facilitate the study by providing a controlled and replicable model, more amenable to mechanistic studies of experimental microbes used to treat CDI. To this end, I challenged mice with varying doses of the hypervirulent VPI10463 *C. difficile* strain notable for hyperproduction of exotoxin. I found that administration of high doses of infectious VPI10463 spores (10^5) would invariably recapitulate fulminant CDI in ASF mice and lead to certain mortality from shock within 3 days unless an effective prophylactic treatment was administered (i.e. a FMT or an effective experimental treatment). Additionally, the extreme susceptibility of the ASF microbiome to infection from CDI meant that very low doses (10^1) would infect mice, however if the mice were

young (approximately 8 weeks of age) and healthy, a majority would not progress to fulminant colitis and instead result in chronic CDI symptoms of moderate severity, including diarrhea, hunched posture and pain, and increased levels of inflammatory cytokines as measured from colon rRNA.

I attempted to use the acute challenge and chronic CDI ASF mouse models to interrogate the mouse fecal microbiome to uncover which taxa play a disproportionate role in protecting their hosts from CDI. A fecal preparation derived from adult mouse feces treated with concentrated chloroform to generate a fecal prep dominated by Clostridia microbes (Clostridia prep), which had been found to protect neonatal mice from mouse pathogens, served as the starting candidate for our studies. Metagenomic sequencing of feces, collected at various time points, tracked changes in the Chronic CDI mouse model microbiome as mice cleared *C. difficile* following administration of the Clostridia prep. This longitudinal tracking of the recovering mouse microbiome made it possible to identify taxa with the strongest negative association with *C. difficile*: the family *Lachnospiraceae*. In-vitro cultivation aimed at maximizing the recovery of *Lachnospiraceae* led to the growth of a bulk culture predominated by *Lachnospiraceae*.

This *Lachnospiraceae* consortium retained efficacy in the ASF mouse models, and the individual species were identified by longitudinal tracking of them in the *Lachnospiraceae* consortium, as associated with removal of *C. difficile*. The strongest association was found with the uncultured bacteria UBA3401, known only from a partially assembled genome gathered from mouse microbiota (Parks et al., 2017). Efforts at targeting culture media and conditions to recover UBA3401 led to co-cultured colonies containing UBA3401. Although efforts to propagate a pure culture were unsuccessful, the colonies, when administered to an ASF host, resulted in colonization by a consortium dominated by UBA3401 (UBA3401 consortium)

accompanied by a small number of microbes, including *Turicibacter. Sp,* possibly required for host colonization. It was found that this defined consortium still retained significant efficacy against challenge with 10^5 VPI10463 which would otherwise lead to fatal fulminant CDI.

Although a pure culture could not be obtained, Hall and O'toole's seminal study demonstrated that even if a microbe of interest cannot be fully isolated, it is still possible to characterize the effects that the microbe and its confederates have upon a host and to determine if the microbe produces metabolites of interest. To do the same with UBA3401, feces from ASF mice colonized by the UBA3401 consortium were examined via metagenomic sequencing and the genomes of the consortium members were screened via bioinformatic software for genes that may be relevant to CDI protection. Notably, the UBA3401 genome appears to contain a functional biosynthetic gene cluster (BGC) for thiopeptide with organization consistent with canonic thiopeptide BGC arrangement. Thiopeptides are known to be narrow spectrum bacteriocins with a spectrum of activity that includes *C. difficile* and offer the potential to attack the pathogen while sparing commensal microbiota (Kim et al., 2022). Efforts to characterize UBA3401 and its likely products are underway. It is hoped to shed light upon the requirements for microbial interventions for CDI, and ultimately lead to more effective noninvasive interventions by identifying taxa involved in resistance against *C. difficile* and its potential mechanisms.

Probiotic interventions with *C. difficile*, although attractive due to their potential as a noninvasive, predictable, cost-effective adjunct to current first line therapies, have been constrained relative to their potential due to the poor efficacy of commercially available probiotics. Key to developing improved microbial therapeutics is the concept that, when it comes to their effect upon a host, bacteria are not equal. Determining which taxa provide

disproportionately strong colonization resistance, and what mechanisms they utilize, will not only allow for more effective species to be selected but also any mechanisms uncovered may lead to the development of new therapies. This study found *Lachnospiraceae* family microbes - already implicated in *C. difficile* colonization resistance - to be sufficient to fully replicate the Clostridia prep's *C. difficile* colonization resistance.

Curiously, a consortium predominated by the microbe UBA3401 and the small number of syntrophic microbes that comprise this consortium was sufficient to impart significant protection. It protected mice administered a lethal dose of V10463 from developing any illness more significant than transient mild CDI. Although not sufficient to fully clear C. difficile, the UBA3401 consortium significantly suppressed the host's C. difficile burden as determined from VPI shed in feces and turned what became fatal fulminant colitis in identical controls, to a transient mild CDI followed by asymptomatic carriage. This demonstrates that a relatively simple, defined consortium may still exert significant protection from CDI and can be used to drive out a pathogen species by maximizing resistance against it by utilizing strategies, like in this study, employing a known bacteriocin producer.

Attempts at cultivating UBA3401 have resulted in limited success thus far: UBA3401 in its first passage appears to grow in blood-enriched UBA3401 selective media. Colonies germinated from spore preparation are PCR positive for UBA3401 and readily colonize mice with a consortium dominated by UBA3401. However, all attempts at subculturing the colonies resulted in no detectable UBA3401 and only the growth of other members of the consortium. This is hypothesized to be due to UBA3401 requiring for its growth a metabolite, likely produced by other members of the consortium. Unlike less fastidious microbes UBA3401 is likely not metabolically self-sufficient and cannot synthesize or convert from primary

metabolites all the nutrients it requires, depending on syntrophic microbes, like many other "unculturable" microbes (Imachi et al., 2022).

Evidence for this hypothesis can be observed in the UBA3401 consortium's difficulty in colonizing germ-free mice when compared to ASF colonized mice – which contain a metagenome fully capable of carbohydrate and energy metabolism (Proctor et al., 2022). Further evidence is provided by longitudinal sequencing of ASF mice administered UBA3401, where engraftment of *Turicibacter. sp* precedes the detection of UBA3401 in its mouse by days. This temporal association is supported by recent findings that the *Turicibacter* genus is highly capable of metabolizing and modifying host bile acids and lipids, producing new molecules (Lynch et al., 2023). Thus, it is consistent with our observed data to hypothesize UBA3401 is likely dependent on the carbohydrate and energy metabolism provided by the ASF microbes, along with lipid products produced by its *Turicibacter* consortium member, in effect a helper for UBA3401. Dependence on the metabolites of other members of a consortium is a significant obstacle to the isolation and study of a microbe but can be resolved by harnessing host and syntrophic microbes to assist in cultivation (Imachi et al., 2022). Notably, bioreactors containing a consortium of microbes generating metabolites that can pass through filters and support the growth of unculturable microbes have shown promise in allowing pure cultures of "unculturable" microbes to be obtained (Imachi et al., 2022). Utilizing a bioreactor replicating intestinal conditions with UBA3401's consortium members could generate filtrate-containing nutrients that would allow UBA3401 to grow in isolation.

The properties and potential of UBA3401 and its consortium remain largely unexplored. Although analysis of the UBA3401 genome suggests UBA3401 possesses a Thiopeptide BGC. Isolation of a product, or even characterization of the putative BGC have yet to be executed.

AntiSMASH analysis of the UBA3401 genome obtained through this study identified a probable Thiopeptide BGC along with many genes belonging to quorum sensing response systems: cyclic lactone autoinducers and ranthipeptides (**Figure 8**). Examination of the region flagged by AntiSMASH found motifs consistent with the biosynthetic requirements of thiopeptide production located in open reading frames (**Figure 9**), including the gene coding for the YcaO enzyme, crucial to thiopeptide biosynthesis (Schwalen et al., 2018; Vinogradov & Suga, 2020). Predicted ABC transporters immediately downstream of the UBA3401 are also characteristic of bacteriocin BGC, where dedicated transporters are located proximate to the BGC and bind and secrete the cytotoxic bacteriocin before it affects the producing cell (Dickey et al., 2023). Follow-up experiments will seek to use primers developed to amplify UBA3401 mRNA products predicted to code for enzymes consistent with canonical genes of a Thiopeptide BGC. Thiopeptide BGC precursors if found, would serve as an indication that the predicted Thiopeptide BGC is indeed functional. Additional follow-up efforts should attempt to use the hydrophobic chloroform extract of mouse fecal matter (demonstrating a limited ability to retard VPI10463 in vitro) for further attempts at fractionation with chromatography. If fractionated extracts maintain in-vitro activity, their chemical composition could be estimated with GC-MS, thereby suggesting which compounds hold direct activity against C. difficile.

Figure 8

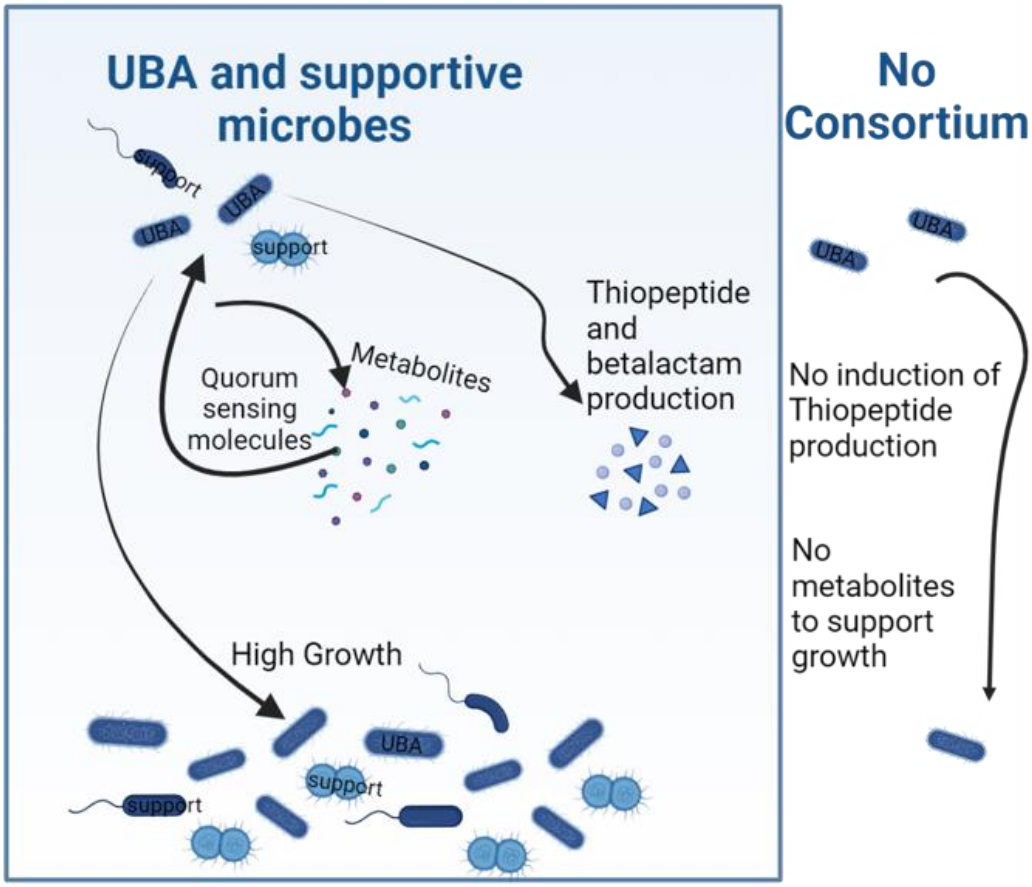


Figure 8. Hypothetical model of UBA3401 quorum-mediated protection against CDI. Hypothesized mechanisms of the UBA3401 consortium's activity against *C. difficile*, and quorum sensing mechanisms governing activity. Discussed in text.

Figure 9

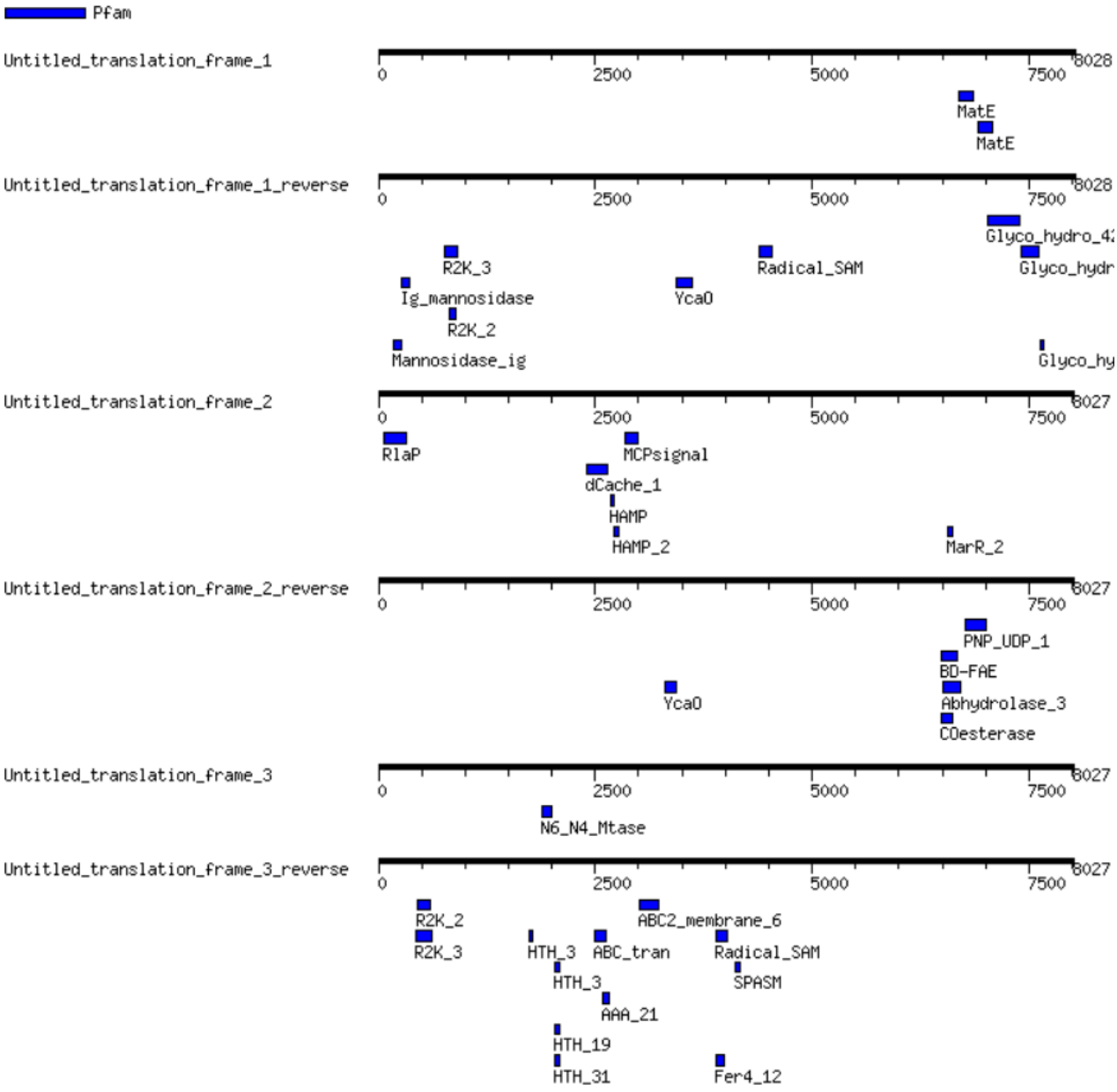


Figure 9: Predicted motifs in UBA3401 thiopeptide BGC. Predicted amino acid sequences of open reading frames from the thiopeptide BGC were screened for motifs via GenomeNet utilizing Pfam's databank.

The UBA3401 genome is also notable for possessing many elements involved in quorum sensing, containing multiple putative cyclic lactone autoinducer and ranthipeptide elements. Cyclic autoinducer peptides serve a key role in gram-positive bacterial quorum sensing, accumulating as the microbes producing them rise in population and inducing signal transduction

(Mull et al., 2018). Indeed, cyclic lactone autoinducing peptides are associated with the induction of bacteriocin production (Michiel Kleerebezem et al., 1997; Sturme et al., 2002), along with production of a host of secondary metabolites in gram-positive microbes. Production of the putative UBA3401 thiopeptide following quorum sensing induction would be consistent with the known functions of cyclic lactone autoinducer genes (Mull et al., 2018; Sturme et al., 2002). Similarly, ranthipeptides, although poorly characterized, have recently been identified as a key element of signal transduction and quorum sensing in Clostridia microbes also inducing phenotypes that only become expressed once a quorum has been reached (Chen et al., 2020).

Taken in context with our other observations, it may be hypothesized that the anti-CDI properties of the UBA3401 consortium are not constitutively active, instead only manifesting upon quorum sensing induction: without a critical threshold of UBA3401 being present, no signal transduction will induce production of thiopeptide or other potential bacteriocins. However, once enough UBA3401 is present, autoinducing cyclic peptides accumulate and signal transduction induces production of thiopeptides. Although the conjectured means of regulating thiopeptide production would not directly require UBA3401's consortium members, they still would be critical to the antimicrobial effect by allowing UBA3401 to grow and reach a quorum. This hypothesis is consistent with our data demonstrating there is a lag between the initial appearance of UBA3401 – Itself appearing only after other consortium members such as *Turicibacter*. *Sp* become present – and its effects against *C. difficile* as UBA3401 grows and reaches a quorum allowing its production of thiopeptide to begin. Future efforts at obtaining the thiopeptide with UBA3401 take into consideration the likely prerequisite of quorum sensing to induce production of its bacteriocins.

Thiopeptides are notable compared to conventional antibiotics for being narrow spectrum with activity against some gram-positive microbes including C. difficile but otherwise sparing most microbes, this makes them particularly attractive against opportunistic pathogens like C. difficile where unlike conventional antibiotics, minimal damage is done to the host microbiome (Hwang et al., 2023; Kim et al., 2022). Once secreted into the cytosol thiopeptides are believed to enter target cells though receptors, with known examples entering through siderophore receptors (Chan & Burrows, 2021, 2023). Once inside the cytosol of a susceptible cell the thiopeptide, depending on the subtype, will inhibit one of the ribosomal elongation factors, aborting peptide elongation and thereby crippling protein translation (Just-Baringo et al., 2014). Large and complex molecular structures hindering de-novo synthesis, and poor water solubility have limited medical use of thiopeptides with only two thiopeptides currently seeing commercial use as veterinary medicines (Just-Baringo et al., 2014), however, the efficacy of thiopeptides against several pathogens, along with their narrow spectrum of action has led to increased interest in the discovery of new molecules (Kim et al., 2019). Ergo, it is of medical interest to ascertain if UBA3401 is producing this bacteriocin.

Classified as ribosomally synthesized and post-translationally modified peptides (RiPPs), Thiopeptides are largely assembled via post-ribosomal peptide synthesis: peptide backbones translated via ribosome are joined and chemically modified by enzymatic machinery to produce a functional protein. Thiopeptides are extensively modified post ribosomally, even compared to other RiPPs, meaning that assembly of a functional thiopeptide is dependent on an array of required enzymes being present (Schwalen et al., 2018). This presents greater complexity compared to a single gene coding for a functional protein not dependent on extensive modification. Ultimately, should future research show UBA3401 indeed produces a thiopeptide,

a way to isolate and produce sufficient thiopeptide to be useful in a therapeutic setting may be to clone the responsible BGC. Cloning of thiopeptide genes, along with identification of the peptide backbones, the modifying enzymes and elements required for function, has been successfully accomplished with thiopeptides from other species (Engelhardt et al., 2010; Liao et al., 2009). Such approaches have been exploited to express the thiopeptide peptide-backbone in species more amenable to culture (Hudson et al., 2015). Expression of the hypothesized thiopeptide peptide-backbone and BGC enzymes in a new host should result in production of the UBA3401 thiopeptide. With the region of interest within the size range of some plasmid vectors, cloning out and assembling the thiopeptide BGC into a plasmid may be possible. Should the thiopeptide BGC successfully express in an easily grown species, mass production of thiopeptide may be possible, potentially allowing for a new noninvasive treatment for mild CDI. Although the water insoluble nature of thiopeptides has stymied their clinical use, hydrogels have been utilized to effectively deliver water insoluble and chemically labile drugs to hosts (Larrañeta et al., 2018). A thiopeptide drug with a narrow spectrum of activity against C. difficile, delivered to the large intestine by hydrogel, would offer a lower relapse risk than first line antibiotics; ergo continued research into UBA3401 and the bacteriocins it potentially yields, is of clinical significance and should be continued.

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Vitae

Education

- Doctor of Philosophy, (August, 2024) Georgia State University, Translational Biomedical Sciences
- Master of Science, (August, 2017) Georgia State University, Biological Science (Microbiology concentration)
- Bachelor of Science, (August, 2014) Georgia State University, Biological Science

Academic Experience:

- PhD Candidate and Research Assistant, Institute for Biomedical Sciences. Georgia State University, Atlanta, GA. (2018 - 2024)
- Lead Tutor, Biology Tutoring Center, Georgia State University, Atlanta, GA. (2016 - 2018)
- Graduate Research Assistant, Institute for Biomedical Sciences. Georgia State University, Atlanta, GA. (2015 - 2017)
- Undergraduate Research Assistant, Department of Biology, Applied and Environmental Microbiology, Georgia State University, Atlanta, GA. (2014 - 2015)

Other Experience and Professional Memberships

- 2011 Certification, BLS for Healthcare Providers, American Heart Association
- 2014 Regular Member, Biological Chemistry Division, American Chemical Society
- 2015 Regular Class, American Association of Bioanalysts
- 2024 American Society for Clinical Pathology Molecular Biology, (Approved pending exam)

Administrative Positions

- GSU, College of Arts and Sciences, Panelist for Graduate Student Panel: Navigating Your Journey from MS Student to Scholar in the Sciences. 2016

Publications

- Prevention and cure of murine C. difficile infection by a Lachnospiraceae strain. Juan Noriega Tejada, William A. Walters, Andrew T. Gewirtz et al. Gut Microbes (accepted pending revisions)
- Western-style diet impedes colonization and clearance of Citrobacter rodentium. Junqing An, Xu Zhao, Yanling Wang, Juan Noriega, Andrew T. Gewirtz , Jun Zou. Plos Pathogens

- Segmented Filamentous Bacteria Prevent and Cure Rotavirus Infection. Zhenda Shi, Jun Zou, Zhan Zhang, Xu Zhao, Juan Noriega, Benyue Zhang, Benoit Chassaing, Andrew T. Gewirtz et al. Cell

Abstracts and presentations

- Use of Altered Schaedler Flora Mice As A Model of Chronic Clostridium Difficile Infection. Presented at: ASM 2017, Atlanta, GA, 2017.
- Construction of a Tetracycline-Inducible System for Controlled Gene Expression in Crithidia fasciculata. Presented at: Georgia State University Undergraduate Research Conference, Atlanta, GA.

Honors and Awards

- Summa cum Laude, Georgia State University, 2014
- Graduation with Distinction, Biology Department, Georgia State University, 2014