ABSTRACT

REED, AMBER DAWN. Contribution of Inhibitory Metabolites and Competition for Nutrients to Colonization Resistance against *Clostridioides difficile* by Commensal *Clostridia.* (Under the direction of Dr. Casey Theriot)

Clostridioides difficile is an anaerobic pathogen that causes significant morbidity and mortality. Understanding the mechanisms of colonization resistance against *C. difficile* is important for elucidating the mechanisms by which *C. difficile* is able to colonize the gut after antibiotics. There are multiple methods of colonization resistance, including the production of inhibitory metabolites and competition for nutrients. Commensal *Clostridia* play a key role in colonization resistance, and those containing the bile acid inducible (*bai*) operon are able to modify bile acids which alter the *C. difficile* life cycle. *C. scindens* and *C. hiranonis* convert cholate (CA) to deoxycholate (DCA) in a rich media, and *C. scindens* is capable of inhibiting *C. difficile* growth which is correlated with the production of ~2mM of DCA and increased expression of *bai* operon genes.

These commensals also compete with *C. difficile* for essential nutrients such as proline and hydroxyproline. In *C. difficile, hypD* is essential for the utilization of hydroxyproline and mice challenged with a Δ*hypD* mutant had reduced weight loss, toxin activity, and an altered microbiota when compared to mice challenged with the WT strain. The transcriptomic response to hydroxyproline is different between *C. difficile, C. hiranonis, C. hylemonae* and *C. scindens,* but expression of genes relating to proline fermentation were elevated in *C. difficile* and some of the commensal *Clostridia* when grown in the presence of hydroxyproline. The strain dependent inhibition of *C. difficile* and the differential responses to hydroxyproline indicates that these commensals should not be treated as a monolith, and that further elucidating the differences between them is important to determining their role in colonization resistance against *C. difficile.*

This approach may allow for the development of rationally designed cocktails of commensal microbes that can compete against *C. difficile* in an infected host.

Contribution of Inhibitory Metabolites and Competition for Nutrients to Colonization Resistance against *Clostridioides difficile* by Commensal *Clostridia*

by
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DEDICATION

This dissertation is dedicated to my partners Paul and Liz. You have both supported me so much through this process and I could not have done it without either of you.

BIOGRAPHY

Amber Reed was born in Pocatello, Idaho and developed a love of science and the natural world at a young age. Learning about the microbial communities surrounding hydrothermal vents kick started her interest in microbiology, and she's been fascinated by microbes ever since. She earned her B.S. of Biology from Guilford College in Greensboro in 2010. She spent some years working as a quality assurance technician in industry before deciding that she wished to pursue a career in research. Under the guidance of Dr. Melanie Lee-Brown at Guilford College, she started a post Baccalaureate research project in the fall of 2015 while continuing to work full time as a laboratory technician. Her work focused on methods of biofilm prevention and dispersal, and she completed her research project in the spring of 2016.

Amber was accepted to the microbiology program at NC State during the fall of 2016, and joined Dr. Casey Theriot's lab. During her time at North Carolina State University, Amber won a T32 Molecular Biotechnology Training Program Fellowship which funded her research for two years. She also served an internship at Novozymes in the summer of 2019 which solidified her desire to work in industry after graduation. Under the direction of Dr. Theriot, her research focused on the gastrointestinal pathogen *Clostridioides difficile* and the role that commensal *Clostridia* play in colonization resistance against the pathogen. The results of her time working in Dr. Theriot's lab are detailed herein.

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On a more personal note, I am grateful for my support network of friends, family and fellow grad students. I know so many amazing and inspiring people and I don't have the space to name them all, but Clara, Cynthia, Laura and Jay have been some of the best friends anyone could ask for. My partner Paul has been cheering me on and supporting my career since before I even considered going to grad school, and Liz has been a source of endless encouragement and boundless energy. Thank you both for believing in me when I could not believe in myself. I could not share my life with finer people.

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CHAPTER 1: Literature Review

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Abstract

Clostridioides difficile is an anaerobic pathogen that causes significant morbidity and mortality. Understanding the mechanisms of colonization resistance against *C. difficile* is important for elucidating the mechanisms by which *C. difficile* is able to colonize the gut after antibiotics. Commensal *Clostridium* play a key role in colonization resistance. They are able to modify bile acids which alter the *C. difficile* life cycle. Commensal *Clostridium* also produce other inhibitory metabolites including antimicrobials and short chain fatty acids. They also compete with *C. difficile* for vital nutrients such as proline. Understanding the mechanistic effects that these metabolites have on *C. difficile* and other gut pathogens is important for the development of new therapeutics against CDI, which are urgently needed

Introduction

Clostridioides difficile is an anaerobic, spore-forming, toxigenic bacterial pathogen that was first isolated from the stool of newborn infants in 1935 [1]. *C. difficile* infection (CDI) is the cause of significant morbidity and mortality and is responsible for over 4.8 billion dollars in excess medical costs yearly [2, 3]. While the current first line treatment of vancomycin is capable of resolving CDI, 20-30% of patients will experience a recurrence within 30 days. Additionally, 40-60% of patients who experience recurrent CDI once will have multiple recurrences [4, 5]. The use of antibiotics, including vancomycin, is a significant risk factor for CDI due to its ability to alter the gut microbiota, resulting in a loss of colonization resistance against *C. difficile* [6-8]. Colonization resistance is defined as the ability of the indigenous gut microbiota to protect against colonization

by pathogens such as *C. difficile* [9]. Understanding the mechanisms of colonization resistance against *C. difficile* is important for determining the mechanisms by which *C. difficile* is able to colonize the gut after antibiotics, and is important for developing new therapeutics and preventatives for CDI. While there are different mechanisms of colonization resistance, there is evidence that commensal gut bacteria from the genus *Clostridium* may play a key role, especially those capable of producing secondary bile acids, which are inhibitory to *C. difficile* [10-14]. In this review we highlight how commensal *Clostridium* found in the gut are able to alter colonization resistance against *C. difficile,* with a particular emphasis on the production of secondary bile acids and other inhibitory metabolites, as well as competition for nutrients.

Primary and secondary bile acids alter the *C. difficile* life cycle

Bile acids are important signaling molecules that modulate various metabolic functions, play an essential role in fat digestion, and help shape the gut microbiota [15, 16]. Primary, or host derived, bile acids are synthesized in the liver from cholesterol in a multistep enzymatic process via the classical or alternative pathway [17, 18]. The classical pathway generates cholate (CA) and chenodeoxycholate (CDCA), whereas the alternative pathway predominately synthesizes CDCA [18]. Primary bile acids as well as secondary bile acids that have gone through enterohepatic circulation are conjugated with either taurine or glycine which makes them impermeable to cell membranes, permitting higher concentrations of bile acids within bile and the gut [19]. These conjugated bile acids are released into the duodenum in response to food ingestion [18, 20]. In the small intestine, conjugated primary bile acids with taurine and or glycine are deconjugated by bile salt hydrolases (BSHs) commonly encoded by gut bacteria [21]. After deconjugation, these primary bile acids are further altered by bacteria in the colon in a myriad of ways to create a diverse pool of secondary, or microbiota derived bile acids. Common secondary bile acids found in the gut

include deoxycholate (DCA) and lithocholate (LCA) which are generated by 7α-dehydroxylation from CA and CDCA, respectively [21, 22]. DCA and LCA can be epimerized by hydroxysteroid dehydrogenases (HDSHs), generating such bile acids as ursodeoxycholate (UDCA), iso-DCA (iDCA) and iso-LCA (iLCA)[23].

Primary and secondary bile acids significantly alter the *C. difficile* life cycle. While bile acids are known to have detergent-like properties that can disrupt bacterial cellular membranes and cause cell lysis, they also affect spore germination, outgrowth and toxin activity of *C. difficile in vitro* at sub-inhibitory concentrations [24-26]. In particular, taurocholate (TCA) is a powerful germinant for *C. difficile* spores (Figure 1) [25]. Primary bile acids glycocholate (GCA) and CA and the secondary bile acid DCA also stimulate spore germination while the primary bile acid CDCA, and the secondary bile acids LCA and UCDA inhibit germination of *C. difficile* spores *in vitro* [24,25,27,28]. Secondary bile acids hyodeoxycholate (HDCA), DCA, iDCA, UDCA, LCA and iLCA decrease the growth of *C. difficile in vitro* in a dose dependent manner (Figure 1) and also reduce toxin activity in some strains of *C. difficile* [24,25,29,30]. While the mechanism of how these bile acids alter *C. difficile* has yet to be fully defined, some progress has been made using proteomic approaches. Specifically, when actively growing *C. difficile* is exposed to sub-inhibitory concentrations of CA, DCA, CDCA or LCA *in vitro*, the abundance of cell wall binding proteins, cellular chaperones, and cell division proteins increase [26]. When *C. difficile* is grown with sub-inhibitory concentrations of CA, DCA, CDCA or LCA for a longer period of time, the abundance of alcohol dehydrogenases AdhE1 and AdhE2 decrease, inhibiting the conversion of acetyl-CoA to butynol or ethanol [26]. This indicates that bile acid stress alters the flux through central metabolic pathways of *C. difficile* as well as causing more generalized stress responses. Bile acids also affect enzymes required for Stickland fermentation, which is required for the growth of *C. difficile* and

several other bacteria in the genus *Clostridium* [27, 28]. Stickland fermentation allows amino acids to be used as an energy source by coupling the oxidation and reduction of paired amino acids to the formation of ATP [29]. Most of the enzymes involved in the reductive Stickland fermentation of leucine to isocaproate increase in abundance when cells are exposed to CA, DCA, CDCA or LCA [26]. The addition of CA or DCA causes an increased abundance of the proline reductase enzymes PrdA, PrdB and PrdC, which are required for Stickland fermentation of proline in *C. difficile*, while the addition of CDCA or LCA causes a decreased abundance of those same three enzymes. This indicates that different bile acids can alter *C. difficile* metabolism. Further studies are needed to clarify how specific bile acids are able to shape the formation and activity of proline reductase enzymes, as well as the effect that the altered expression of Stickland fermentation enzymes has on the competitive fitness of *C. difficile.*

Select bile acids can also induce morphological changes in *C. difficile* cells. CDCA, DCA and LCA cause a significant decrease in the presence of flagella, as well as the flagellar structural protein FliC, and flagellar filaments disappear almost entirely when *C. difficile* is challenged with LCA *in vitro* [26]. In addition, bacterial cells challenged with CA, DCA or CDCA were significantly longer than the untreated cells, which is an indicator of bacterial stress, but the addition of LCA does not affect cell shape [26]. DCA causes a significant increase in biofilm formation by *C. difficile in vitro,* whereas LCA does not impact biofilm formation[30]. While CDCA, DCA and LCA are able to impact toxin activity in *C. difficile in vitro,* the mechanism was unknown until recently [24, 31]. Bile acids, including DCA and LCA, bind in a reversible fashion to TcdB, one of the two primary toxins carried by *C. difficile* [31]. LCA and CDCA are able to bind to TcdB with high efficiency and they are able to inhibit cell rounding, a sign of cell death, in human fibroblast cells[31]. DCA binds to TcdB with lower efficiency than LCA and CDCA, and does not inhibit cell rounding in human

fibroblasts [31]. This binding induced a major conformational change in TcdB, which inhibited the ability of the toxin to bind cell surface receptors of HCT 116 cells, a human colonic cell line[31]. This mechanistic *in vitro* work demonstrates that bile acids elicit dynamic effects on *C. difficile* and manipulation of the bile acid pool could be a promising therapeutic strategy for treating CDI.

Secondary bile acids are also associated with protection against CDI in mouse models and human subjects [7,10,36]. An increase in primary bile acids and a loss of secondary bile acids is observed after treatment with antibiotics and is associated with increased susceptibility to CDI [7, 8, 32-36]. Cecal extracts from mice made susceptible to CDI stimulate *C. difficile* spore germination, while cecal extracts from mice resistant to CDI inhibit spore germination, indicating that antibiotic induced changes in bile acid levels *in vivo* are sufficient to induce germination and outgrowth of *C. difficile* spores [36, 37]. However, *C. difficile* spores are able germinate in the small intestine prior to antibiotics, indicating that the bile acids present in the small intestine do not protect against CDI[37]. After human fecal microbiota transplantation (FMT), an increase in microbial diversity is observed and secondary bile acid metabolism is restored[38, 39]. Specifically, the levels of secondary bile acids including DCA, LCA and UCDA are increased and the primary bile acids CA and CDCA are decreased in CDI patients after receiving an FMT [39]. In addition, fecal samples of patients with CDI have a lower prevalence of *baiCD,* a gene present in commensal *Clostridium* required for the synthesis of DCA and LCA via 7α-dehydroxylation, although *baiCD* has also been found in the stool samples of individuals with failed FMTs [12, 40]. *Clostridium scindens* is a commensal bacterium found in the gut microbiota [41]. It produces DCA and LCA and is associated with the return of colonization resistance against *C. difficile* in a mouse model of CDI, however *C. scindens* has also been found to be present in the stool samples as of individuals with CDI [10, 42]. Mice that receive *C. scindens* before being challenged with *C. difficile* show increased levels of LCA,

although levels of most other bile acids are unchanged [10]. While manipulation of the bile acid pool using commensal bacteria is a promising strategy, the addition of exogenous bile acids can also affect the progress of CDI. Challenging mice exogenously with the secondary bile acid UDCA attenuates disease early during CDI, and also alters the fecal bile acid metabolome without significantly altering the gut microbiome [43].

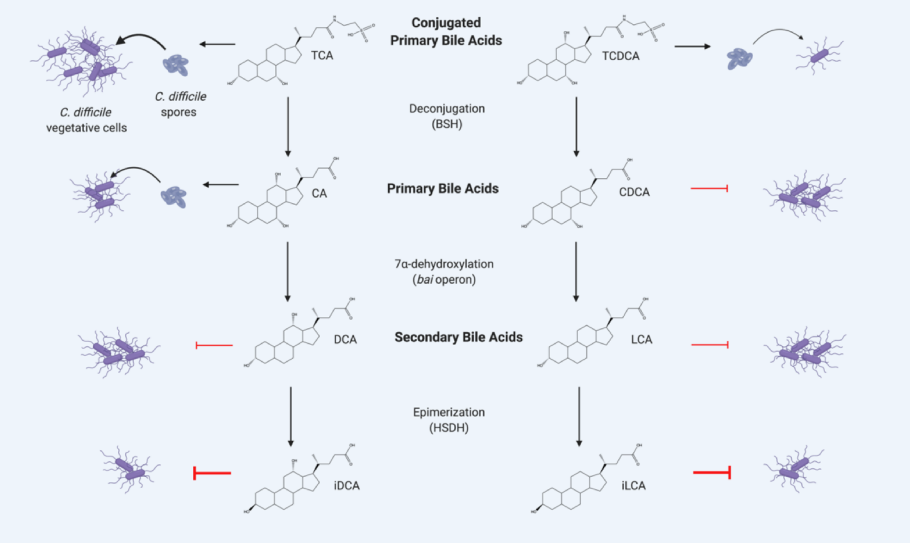

Figure 1: Selected transformations of bile acids carried out by gut bacteria and their effect on *C. difficile.* TCA is a strong germinant for *C. difficile* spores and TCDCA is a weak germinant. CA is a moderate germinant for *C. difficile* spores, and CDCA inhibits vegetative *C. difficile.* The secondary bile acids DCA and LCA inhibit vegetative *C. difficile* and iDCA and iLCA strongly inhibit *C. difficile.* Abb. BSH, bile salt hydrolase; *bai,* bile acid inducible; HSDH, hydroxysteroid dehydrogenase; TCA, taurocholate; TCDCA, taurochenodeoxycholate; CA, cholate; CDCA, chenodeoxycholate; DCA, deoxycholate; LCA, lithocholate; iDCA, iso-deoxycholate; iLCA, isolithocholate. Created with BioRender.com

Bile acid altering enzymes encoded by commensal *Clostridium*

Bile salt hydrolases

Bile salt hydrolases (BSHs) are microbial enzymes that deconjugate primary and secondary bile acid from the amino acids they are conjugated to, usually taurine and glycine[44]. While BSHs are commonly encoded by multiple members of the gut microbiota, commensals in the genus

Clostridium rarely encode BSHs, although *C. hiranonis* and the pathogen *Clostridium perfringens* both encode BSHs and have demonstrated BSH activity [21, 45]. *C. hiranonis* is the only bacterium to date known to have the capability for both 7α-dehydroxylation and deconjugation[45]. The presence of BSHs in the gut are hypothesized to be important for several reasons. BSHs are considered the gateway step for the transformation of primary bile acids to secondary bile acids, as further transformations cannot occur until the conjugated amino acid is removed [21]. The taurine or glycine that is released when deconjugation occurs may be acquired for nutrition by members of the gut microbiota [46]. A recent study showed that bile acids can also be conjugated with tyrosine, phenylalanine or leucine in mice, however deconjugation of these conjugated bile acids by BSHs is unknown at this time [47]. Interestingly, one strain of an unnamed *Clostridium* bacterium capable of deconjugation shows increased growth when taurine was added to the growth medium, indicating that BSH activity might be nutritionally beneficial [48]. Taurine is also enriched in the feces of pediatric inflammatory bowel disease patients with CDI, indicating a potential association between *C. difficile* and taurine [49].

In addition, a *bsh* encoded by *Bifidobacterium longum* is transcriptionally coupled to *glnE* (glutamine synthetase adenylyltransferase), which indicates that deconjugation activity may be coupled to nitrogen regulation [50]. However, lactobacilli grown with conjugated bile acids do not utilize the steroid moiety of the bile acid for cellular precursors and taurine does not affect growth, indicating that not all bacteria encoding a *bsh* obtain a direct nutritional benefit from deconjugation [51]. BSH activity has been hypothesized to detoxify conjugated bile acids by converting them to a less toxic form, as the *bsh* encoded by *Listeria monocytogenes* is important for resistance to bile *in vitro* and is an important virulence factor in animal models [52]. However, unconjugated bile acids are more toxic to some *Lactobacillus* spp. than their conjugated forms, meaning that deconjugation

of bile acids can cause an increase in toxicity for at least some members of the gut microbiota [53, 54]. Conjugated bile acids are more soluble than deconjugated bile acids, so the increased toxicity observed may be offset by the decreased bioavailability that occurs when micelles form[55, 56]. In addition, deconjugation is important for producing free bile acids available for 7α-dehydroxylation [41, 57]. BSH activity is also correlated with resistance to *C. difficile* after FMT[58]. Pre-FMT stool samples harbor reduced BSH activity and a lower proportion of BSH-producing bacterial species when compared with donor stool and post-FMT stool. Additionally, mice inoculated with *Escherichia coli* expressing a highly active BSH have a ~70% reduction in *C. difficile* viable counts when compared to mice inoculated with non-BSH expressing *E. coli* [58]. This indicates that BSH activity could be a significant contributor to the efficacy of FMT in treating recurrent CDI.

Bile acid inducible operon

Commensal *Clostridium* that harbor the bile acid inducible (*bai*) operon are capable of synthesizing DCA from CA and LCA from CDCA via 7α-dehydroxylation. While the enzymes responsible for the steps in the oxidative arm of the metabolic pathway have been known for some time, the reductive arm has only recently been defined by reconstructing the pathway *in vitro* [59]. The proton-dependent transporter BaiG is responsible for transporting the primary unconjugated bile acid into the cell [60]. Six core enzymes encoded by the *bai* operon are sufficient for completing the 7α-dehydroxylation pathway [59]. Coenzyme A is ligated onto the substrate in an ATP dependent manner by BaiB and the dehydrogenase BaiA2 oxidizes the 3-hydroxy group [61, 62]. The NADH:flavin-dependent oxidoreductase BaiCD catalyzes the formation of the C_4=C_5 bond and the 7α-dehydratase BaiE catalyzes the formation of the C_6=C_7 bond by removing the 7α hydroxyl group [12, 63]. The 7α-dehydration is the last step in the oxidative arm of the pathway and is

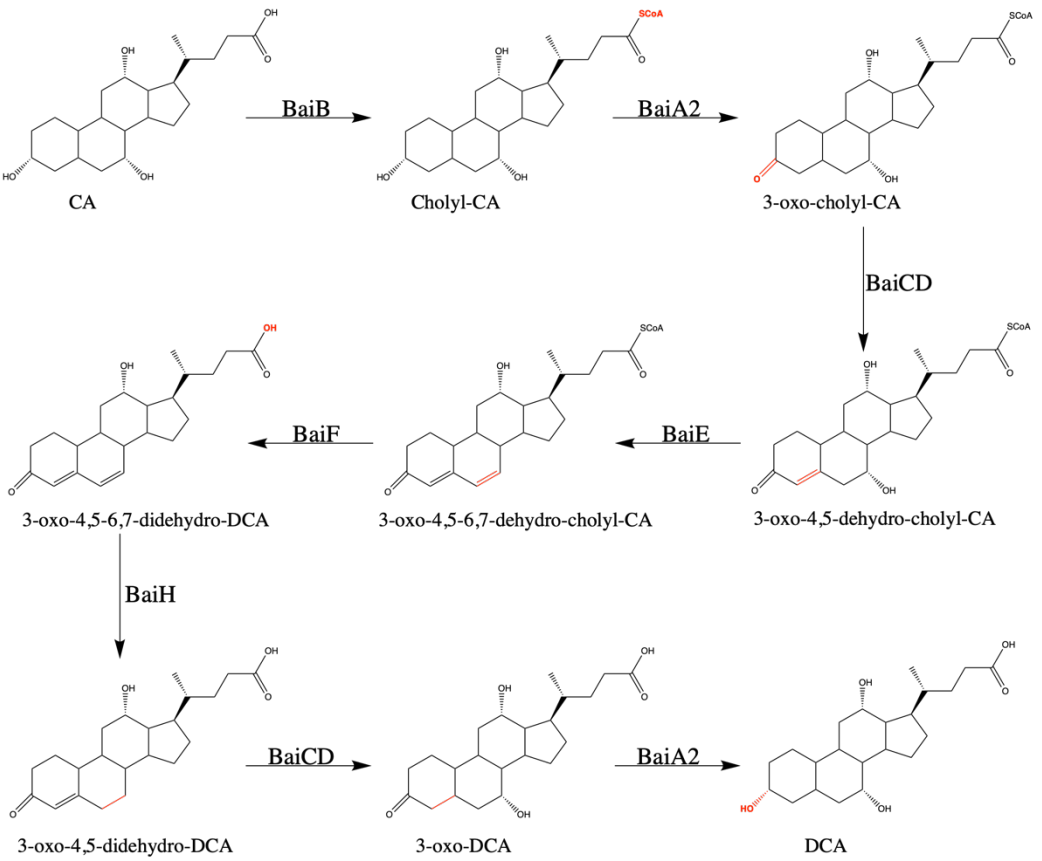

Figure 2: Proposed pathway for conversion from CA to DCA via 7α-dehydroxylation. This metabolic pathway converts CA to DCA or converts CDCA to LCA in eight steps. Abb. BaiB, bile acid-coenzyme A ligase; BaiA2, 3α-hydroxysteroid dehydrogenase; BaiCD, 7α-hydroxy-3-oxo-Δ^4-cholenoic acid oxioreductase; BaiE, bile acid 7α-dehydratase; BaiF, bile acid coenzyme A transferase/hydrolase; BaiH, 7β -hydroxy-3-oxo-Δ^4-cholenoic acid oxioreductase; CA, cholate; DCA, deoxycholate.

irreversible and rate limiting [64]. The reductive arm of the 7α-dehydroxylation consists of four steps. The removal of Coenzyme A is catalyzed by the bile acid-CoA hydrolase BaiF, which can also ligate CoA onto the primary unconjugated bile acid in an ATP independent manner [65, 66]. The NADH:flavin-dependent oxidoreductases BaiH and BaiCD catalyze the removal of the C_6=C_7 and C_4=C_5 bonds and BaiA2 performs the final reductive step, catalyzing the transformation from 3-oxo-DCA to DCA [59]. The enzyme responsible for transport of DCA out of the cell has yet to be determined.

While the enzymes discussed above can sufficiently execute 7α-dehydroxylation, other enzymes are also capable of performing steps in this pathway, indicating some redundancy. Of particular interest is BaiA. While BaiA2 was the enzyme used to reconstruct the 7α-dehydroxylation pathway *in vitro*, another 3α-hydroxysteroid dehydrogenase called BaiA1 is also present in some of the bacteria that have demonstrated 7α-dehydroxylation capability [61]. BaiA1 is a close homolog of BaiA2 with 92% sequence identity that can also perform the oxidative step in the pathway [61]. BaiA1 has not been shown to catalyze the transformation from 3-oxo-DCA to DCA, but since *Clostridium hylemonae* TN 271 carries *baiA1,* but lacks *baiA2* and has been shown to produce DCA, BaiA1 is likely able to perform both steps in the pathway, as *C. hylemonae* would be unable to produce DCA if *baiA2* was necessary for 7α-dehydroxylation [67]. *Clostridium hiranonis* TO 931 carries *baiA2,* but lacks *baiA1,* while *C. scindens* ATCC 35704 and *C. scindens* VPI 12708 carry both [21]. *C. scindens* VPI 12708 also has a second copy of *baiA1,* referred to as *baiA3*[68]. Another enzyme that is capable of performing steps in the 7α-dehydroxylation pathway is the flavoprotein BaiN, which is capable of converting 3-oxo-4,5-6,7-didehydro-DCA to 3-oxo-4,5-dehydro-DCA and then to 3-oxo-DCA, which are steps that can also be performed by BaiH and BaiCD, respectively [59, 69].

While all organisms known to carry the *bai* operon have 7α-dehydroxylation activity, the regulation of the *bai* operon has yet to be fully elucidated [45,67,70,71]. *C. scindens* and *C. hylemonae* have increased expression of genes in the *bai* operon in defined media supplemented with CA, and *C. hiranonis* in rich media supplemented with CA [70, 71]. While *C. scindens* also has increased expression of selected *bai* operon genes when grown in rich media, *C. hylemonae* does not, indicating differences in regulation of the *bai* operon between commensal *Clostridium* [57].

While most of the enzymes involved in 7α-dehydroxylation are not extensively characterized, BaiA and BaiE have both undergone structural and functional characterization [61, 72]. The short chain dehydrogenase/reductase BaiA2 as well as the homolog BaiA1 shows exclusive preference for the cofactor NAD(H) rather than NADP(H), likely due to steric hindrance involving Glu42 in the cofactor binding site [61]. The dehydratase BaiE shows a preference for 3-oxo-Δ^4-CDC-CoA over 3-oxo-Δ^4-CDCA, with the K_{cat}/K_M being an order of magnitude higher for the former than the latter, indicating that the 7α-dehydration step is more efficient when the intermediate is ligated to CoA [72].

Hydroxysteroid dehydrogenases

Bacterial hydroxysteroid dehydrogenases (HSDHs) epimerize bile acid hydroxy groups on the 3-, 7-, or 12- carbons of bile acids in a two-step process requiring an α- and a β-HSDH that generates a stable oxo intermediate [21]. Commensal *Clostridium* can encode multiple HSDHs. Commensal *Clostridium* that encode the *bai* operon carry both a 7α-HSDH (*baiA*) as well as a 7β-HSDH [21]. Organisms with a 7α- and a 7β-HSDH can produce UDCA, which is the 7*β*-epimer of CDCA [21]. As UDCA is more hydrophilic and thus less toxic to gut bacteria than CDCA, the epimerization of CDCA using a 7β-HSDH could serve as a survival advantage for bacteria capable of accomplishing this transformation [21, 73]. In addition, *Ruminococcus gnavus* carries a 3α-HSDH and *C. innocuum* carries a 3β-HSDH [21, 44, 74]. The 3α/β epimerization of DCA and LCA creates iDCA and iLCA, respectively, which are the second most abundant secondary bile acids after DCA and LCA [22]. While no bacteria in the genus *Clostridium* has made iDCA, *R. gnavus* uses a 3α-HSDH to create the intermediate of 3-oxoDCA and then a 3β-HSDH to complete the transformation from DCA to iDCA [74]. iDCA exhibits reduced toxicity *in vitro* to some gut commensals including multiple species of *Bacteroides* and *Clostridium sporogenes,* but has the

ability to inhibit multiple strains of *C. difficile* at very low concentrations [24, 74]. This indicates that the conversion from DCA to iDCA can serve to reduce toxicity for some commensals, as well as assisting the gut microbiota with colonization resistance against pathogens such as *C. difficile.* These same 3α- and 3β-HSDHs convert LCA to iLCA, which inhibits the growth of multiple strains of *C. difficile in vitro* at a lower concentration than LCA [24, 75]. The toxicity of iLCA when compared to LCA on various commensals has yet to be determined, but it is possible that the epimerization of LCA to iLCA serves as to reduce toxicity for some members of the gut microbiota, as well as assisting with colonization resistance against enteric pathogens such as *C. difficile.* In the human gut, UDCA is also the product of epimerization. Specifically, it is the 7*β*-epimer of CDCA [21]. As UDCA is more hydrophilic and thus less toxic to gut bacteria than CDCA, the epimerization of CDCA using a 7β-HSDH could serve as a survival advantage for bacteria capable of accomplishing this transformation [21, 73].

Bile acids, other intestinal pathogens, and the host

While bile acids modified by commensal *Clostridium* affect the life cycle of *C. difficile,* they also have an inhibitory effect on other intestinal pathogens as well as a strong effect on the host. Bile acids can induce the transcription of genes responsible for DNA repair and recombination in *E. coli*, *Salmonella enterica* serovar Typhimurium, *Bacillus cereus*, and *L. monocytogenes* [76, 77]. Genes responsible for maintaining the integrity of the cellular envelope are also upregulated in *B. cereus* and *L. monocytogenes*, indicating that bile acids damage the bacterial membrane and cellular DNA [77]. In particular, multiple strains of *Shigella* show a significant increase in biofilm formation and 143 genes have differential transcription when exposed to bile salts which indicates a strong stress response [78]. Enteric pathogens have multiple bile resistance

mechanisms including efflux pumps and DNA repair mechanisms, but bile acids are still important in colonization resistance against these intestinal pathogens [77].

Bile acids are important signaling molecules within the host as well. They interact primarily with the G-Protein-Coupled Bile Acid Receptor-1 (GPBAR-1, aka TGR5) and Farnesoid-X-Receptor alpha (FXRα) which belong to the nuclear receptor superfamily [17,18,21]. Secondary bile acids produced by commensal *Clostridium* are potent agonists for TGR5, specifically DCA and LCA [17, 79]. TGR5 has been implicated in the regulation of multiple metabolic functions including glucose metabolism and the conversion of fat into energy, making it a potential target for treating obesity [17, 79]. The most potent agonist for FXRα is CDCA, but DCA and LCA are also agonists for this receptor [17]. FXRα controls the enterohepatic circulation of bile acids and acts as an anti-inflammatory mediator in the liver and intestine, which could allow it to potentially help prevent tumor development [17]. However, high levels of the secondary bile acids DCA and LCA have been shown to correlate with tumors in the liver and intestine, specifically colon cancer [17, 21]. High levels of DCA are also correlated with cholesterol gallstone disease in some patients [21]. The levels of bile acids can also affect FXR receptor expression, as giving mice exogenous UCDA increases the expression of TGR5 and FXR, causing alterations to the bile acid metabolome[43].

Bile acids are important not just for their effect on the gut microbiota and their contribution to colonization resistance against *C. difficile* and other intestinal pathogens, they are also important determinants of several other aspects of human health. Further studies examining the rational manipulation of bile acid pools and the effect of this alteration on colonization resistance against *C. difficile* and other intestinal pathogens are necessary, and understanding the production of secondary bile acids by commensal *Clostridium* and other microbes is important for advancing our knowledge of human health and disease.

Production of inhibitory metabolites

While bile acids play a significant role in modulating the composition of the gut microbiota there are other bacterial metabolites that can affect the gut microbiota and colonization resistance against *C. difficile* such as short-chain fatty acids (SCFAs) [38]. SCFAs are metabolized from fiber by commensal bacteria and the concentration of SCFAs are low in patient stool after taking broad spectrum antibiotics, in CDI patients, and in CDI susceptible mice [38, 80]. Increased levels of SCFAs are correlated with decreased tissue damage and immunomodulatory effects, making rational manipulation of SCFA production a potential strategy for targeted therapeutics against CDI. Increased levels of the SCFAs propionate, succinate, and butyrate were observed after FMT for recurrent CDI[38]. In addition, valerate inhibits *C. difficile* in a chemostat model, and butyrate can protect against *C. difficile* induced colitis in the murine gut via reducing intestinal permeability and microbial translocation in an HIF-1 dependent fashion [42,82,83]. Members of the *Clostridium* cluster XIVa and IV are a significant source of butyrate production in the gut, and are significantly depleted in the feces of patients with CDI or with nosocomial diarrhea (*C. difficile* negative) when compared to healthy control samples [81-83].

Despite the ability of multiple strains of *C. difficile* to generate butyrate, the presence of butyrate in the gut is associated with decreased fitness for *C. difficile*[84]. Specifically, when mice are fed microbiota-accessible carbohydrates, the SCFAs propionate, acetate and butyrate increase, and the *C. difficile* burden decreases[84]. In addition, all three SCFAs negatively affect the growth of *C. difficile,* although all three SCFAs cause toxin expression to increase *in vitro* [84]. However, the overall level of toxin decreases due to the lower *C. difficile* burden in mice that are fed diets rich in microbiota-accessible carbohydrates [84].

Butyrate can be produced by bacteria through multiple pathways. The most common pathway in *Clostridium* is the synthesis of butyryl-CoA from acetyl-CoA and the subsequent liberation of butyrate from the CoA molecule[85]. There are multiple arrangements of the butyrate synthesis genes in *Clostridium,* with two arrangements being present in Cluster XIVa and a third distinct arrangement being present in butyrate producing *Clostridium* in Cluster I and Cluster XVI[85]. After butyryl-CoA is produced, the CoA moiety can be removed by butyryl-CoA:acetate CoA transferase (But) or the butyryl-CoA can be phosphorylated by phosphate butyryltransferase (Ptb) then transformed to butyrate by butyrate kinase (Buk) which generates ATP[86]. Most butyrate producing *Clostridium,* including *C. difficile* contain Buk, some contain But instead, and a small number of strains encode both proteins[86].

However, lysine, glutarate, 4-aminobutyrate, and succinate can also serve as substrates for the production of butyrate. These three pathways are separate from the acetyl-CoA pathway, but all four pathways merge at the energy generating step where crotonyl-CoA is transformed into butyryl-CoA by the Bcd complex [86]. Multiple strains of *C. difficile* can generate butyrate using acetyl-CoA, 4-aminobutyrate or succinate as a substrate. *Clostridium sticklandii* can use acetyl-CoA or lysine as a substrate [86, 87]. The generation of butyrate from succinate by *C. difficile* is of particular interest as the ability to ferment succinate gives *C. difficile* a competitive advantage [87].

Antimicrobial compounds produced by members of the gut microbiota also affect colonization resistance against *C. difficile. C. scindens* ATCC 35704 produces 1-acetyl-β-carboline, a tryptophan derived antibacterial compound that inhibits multiple Gram-positive pathogens found in the gut, including *C. difficile, Staphylococcus aureus* and *Clostridium sordellii*[88]. While the specific mechanism of action is not known, cell division of *C. difficile* was

inhibited and the additional presence of DCA or LCA and enhanced the inhibitory effect of 1-acetyl-β-carboline *in vitro* [88].

Competition for nutrients

Competition for nutrients also plays an important role in colonization resistance against *C. difficile* and other pathogens. Colonization of a susceptible murine host by a nontoxigenic strain of *C. difficile* protects against colonization by toxigenic *C. difficile,* indicating that colonization by bacteria with similar nutritional requirements can protect the host[89, 90]. Strains of *C. difficile* belonging to the epidemic ribotypes (RT) 027 and 078 have gained the ability to metabolize low concentrations of trehalose, a common food additive [91]. In the RT 027 strain, a point mutation occurred that increased sensitivity to trehalose while the RT 078 strain acquired additional genes that metabolize trehalose [91]. While the exact contribution to competitive fitness is unknown, the ability to metabolize trehalose increased virulence in a mouse model of *C. difficile,* indicating that increased ability to compete for trehalose in the gut may provide some form of competitive advantage [91]. *C. difficile* also uses sugar alcohols such as mannitol, N-acetylated amino acids, and carbohydrates during early infection in the murine gut, but the effect of each of those nutrients on competitive fitness is unknown [92].

Proline, hydroxyproline and glycine are the most efficient electron acceptors for Stickland fermentation, while leucine, isoleucine and alanine are the most efficient electron donors [27]. *C. difficile* is auxotrophic for isoleucine, leucine and proline, and proline concentration affects the *in vitro* expression of genes in the *prd* operon which is responsible for proline reduction in Stickland fermentation [27, 93]. Availability of these amino acids (alanine, glycine, leucine, isoleucine and proline) in the gut correlates with increased susceptibility to CDI in a mouse model [94]. Proline in particular is important for *C. difficile* colonization as a *prdB* mutant is unable to use proline as an

energy source. When a *C. difficile prdB* mutant was tested in a mouse model of CDI, the mice challenged with the *prdB* mutant had reduced colonization and a lower concentration of TcdB in their stool when compared to mice challenged with wild type *C difficile,* indicating that the ability to ferment proline is important for colonization and virulence [94]. In addition, when wild type *C. difficile* capable of fermenting proline and a *prdB* mutant were grown in the presence or absence of a commensal clostridia panel*,* the wild type *C. difficile* had a fitness advantage when the commensals were present, indicating that the presence of commensal clostridia increases reliance on proline fermentation [95]. However, when *C. difficile* competed with *Paeniclostridium* spp. or *Clostridium xylanolyticum,* two members of the commensal clostridia panel able to ferment proline, the competitive advantage conferred by wild type *C. difficile* in comparison to the *prdB* mutant was lower than when it was only competing with bacteria unable to ferment proline [95]. This indicates that *C. difficile* competes with commensal clostridia for proline. *C. difficile* also has a competitive advantage over *C. scindens, C. hylemonae* and *C. hiranonis* in a rich media, although the extent to which this is due to the ability of *C. difficile* to ferment proline is unknown [57].

Hydroxyproline (Hyp) is a derivative of proline which has been post translationally modified by prolyl-4-hydroxylase [96]. It is important for stabilizing the triple helix structure in collagen, the most abundant mammalian protein [97]. It can be converted to proline in a two-step process that requires the hydroxyproline dehydratase HypD as well as the pyrroline-5-carboxylate reductase ProC, both of which are present in *C. difficile* [98, 99]. Homologs of HypD are widespread in the gut microbiome, which suggests that the ability of bacteria to reduce hydroxyproline is useful in the gut [98]. However, of the bacteria encoding *hypD,* only a subset had an adjacent *proC* gene, indicating that the ability to reduce hydroxyproline to proline is not ubiquitous [98]. While Stickland fermentation of proline is important for *C. difficile* metabolism, it is not yet known how the

reduction of hydroxyproline affects competitive fitness. However, the widespread presence of HypD and the competitive fitness advantage gained by proline fermentation in the presence of commensal clostridia indicates that it may play a significant role in the colonization of *C. difficile* in the gut [95, 98].

Conclusion

There are several mechanisms of how the gut microbiota provides colonization resistance against *C. difficile* presented in this review, including the production of inhibitory metabolites such as secondary bile acids, SCFAs and antimicrobials as well as competition for nutrients, especially proline and other amino acids necessary for Stickland fermentation. Understanding the mechanistic effects that these metabolites have on *C. difficile* and other gut pathogens is important for the development of new therapeutics against CDI, which are urgently needed.

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Chapter 2: Strain-Dependent Inhibition of *Clostridioides difficile* by Commensal *Clostridia* Carrying the Bile Acid-Inducible *(bai)* Operon

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Abstract

Clostridioides difficile is one of the leading causes of antibiotic-associated diarrhea. Gut microbiota-derived secondary bile acids and commensal *Clostridia* that encode the bile acid inducible (*bai*) operon are associated with protection from *C. difficile* infection (CDI), although the mechanism is not known. In this study we hypothesized that commensal *Clostridia* are important for providing colonization resistance against *C. difficile* due to their ability to produce secondary bile acids, as well as potentially competing against *C. difficile* for similar nutrients. To test this hypothesis, we examined the ability of four commensal *Clostridia* encoding the *bai* operon (*C. scindens* VPI 12708, *C. scindens* ATCC 35704, *C. hiranonis,* and *C. hylemonae*) to convert CA to DCA *in vitro,* and if the amount of DCA produced was sufficient to inhibit growth of a clinically relevant *C. difficile* strain. We also investigated the competitive relationship between these commensals and *C. difficile* using an *in vitro* co-culture system. We found that inhibition of *C. difficile* growth by commensal *Clostridia* supplemented with CA was strain-dependent, correlated with the production of ~2 mM DCA, and increased expression of *bai* operon genes. We also found that *C. difficile* was able to outcompete all four commensal *Clostridia* in an *in vitro* co-

culture system. These studies are instrumental in understanding the relationship between commensal *Clostridia* and *C. difficile* in the gut, which is vital for designing targeted bacterial therapeutics. Future studies dissecting the regulation of the *bai* operon *in vitro* and *in vivo* and how this affects CDI will be important.

Importance

Commensal *Clostridia* encoding the *bai* operon such as *C. scindens* have been associated with protection against CDI, however the mechanism for this protection is unknown. Herein, we show four commensal *Clostridia* that encode the *bai* operon effect *C. difficile* growth in a strain-dependent manner, with and without the addition of cholate. Inhibition of *C. difficile* by commensals correlated with the efficient conversion of cholate to deoxycholate, a secondary bile acid that inhibits *C. difficile* germination, growth, and toxin production. Competition studies also revealed that *C. difficile* was able to outcompete the commensals in an *in vitro* co-culture system. These studies are instrumental in understanding the relationship between commensal *Clostridia* and *C. difficile* in the gut, which is vital for designing targeted bacterial therapeutics.

Introduction

Clostridioides difficile is an anaerobic, spore forming, toxigenic bacterial pathogen [1]. *C. difficile* infection (CDI) is a major cause of antibiotic associated diarrhea and a significant health issue, causing 453,000 infections and is associated with 29,000 deaths and 4.8 billion dollars in excess medical costs a year in the U.S. alone [2]. While the current first line treatment of vancomycin can resolve CDI, 20-30% of patients who successfully clear the infection experience recurrence (rCDI) within 30 days, and 40-60% of those who experience one episode of rCDI will experience further recurrences [3, 4]. Antibiotic use is a significant risk factor for CDI, as antibiotics alter the gut microbiome, causing a loss of colonization resistance against *C. difficile* [5-7]. This alteration of

the microbiome also affects the gut metabolome, causing a loss in beneficial metabolites, including secondary bile acids generated by the gut microbiota [6, 8]. Many of these secondary bile acids are inhibitory to *C. difficile in vitro* and are associated with protection against CDI in mice and humans [9, 10].

Deoxycholate (DCA) is an abundant secondary bile acid in the gut, with concentrations ranging from 0.03-0.7 mM in the non-antibiotic treated gut [11]. DCA is synthesized from the primary bile acid cholate (CA) via a multistep pathway that results in 7α-dehydroxylation of CA [12]. The enzymes responsible for this synthesis are encoded in the bile acid inducible (*bai*) operon, which is also capable of synthesizing the secondary bile acid lithocholate (LCA) from the primary bile acid chenodeoxycholate (CDCA) [13]. A small population of commensal bacteria encoding the *bai* operon are capable of transforming CA to DCA, including *Clostridium* cluster XIVa members *Clostridium scindens* and *Clostridium hylemonae* as well as *Clostridium* cluster XI member *Clostridium hiranonis* [14-16]. Several enzymes are capable of completing the steps in this transformation, including the bile acid transporter BaiG, the bile acid 7α-dehydratase BaiE, and the flavoprotein BaiN [13, 17-19]. While regulation of the *bai* operon and of *baiN* has yet to be completely elucidated, *in vitro* studies show that CA upregulates genes in the *bai* operon and DCA downregulates them in *C. scindens* ATCC 35704, *C. hylemonae,* and *C. hiranonis* [20, 21].

Secondary bile acids are able to inhibit different stages of the *C. difficile* lifecycle. DCA alone is able to inhibit the outgrowth of *C. difficile,* reduce motility and decrease expression of flagellar proteins and toxins *in vitro* [9, 22-24]. *In vivo* studies show the presence of *baiCD,* a gene needed for 7α-dehydroxylation, is negatively correlated with CDI in humans, although in another study *C. scindens* was present in the same stool samples as *C. difficile* [25, 26]. In addition, *C. scindens* ATCC 35704 is associated with protection against CDI in mouse models, and *C. hiranonis* is

negatively correlated with the presence of *C. difficile* in canines, but the exact mechanism of this potential protective effect is still unknown [27-29].

While the production of inhibitory metabolites such as secondary bile acids may be responsible for these potential protective effects, competition for nutrients from other bacteria in the gut, including commensal *Clostridia,* may also play a role. Nutrient competition is another mechanism by which the gut microbiota provides colonization resistance against pathogens. A decrease in specific gut metabolites that *C. difficile* requires for growth (e.g. proline, branched chain amino acids, and carbohydrates) have been associated with CDI in a mouse model and in humans [30-32]. In support of this, colonization of a susceptible host by a non-toxigenic strain of *C. difficile* can protect against later colonization by a toxigenic strain [33, 34]. This suggests that colonization by a bacterial strain with similar nutritional requirements can have a protective effect on the host. There is evidence that *C. difficile* shares some nutritional requirements with commensal *Clostridia*, including the amino acid tryptophan and the vitamins pantothenate and pyridoxine, which both *C. scindens* ATCC 35704 and *C. difficile* are auxotrophic for [21, 35]. *C. difficile* is also auxotrophic for five additional amino acids other than tryptophan, including isoleucine, leucine and proline which are all highly efficient electron donors or acceptors in Stickland fermentation [35, 36]. Products of Stickland fermentation are important for growth in *C. difficile* and many other *Clostridia* such as *Clostridium sticklandii* and *Clostridium sporogenes* [35-40]. *C. hiranonis* and *C. hylemonae* both contain genes encoding for enzymes involved in Stickland fermentation that were highly expressed *in vivo. C. scindens* ATCC 35704 has also demonstrated *in vitro* genomic potential for Stickland fermentation [20, 21]. Therefore, these commensal *Clostridia* could potentially compete with *C. difficile* for the amino acids it requires for growth and colonization.

In this study, we hypothesized that commensal *Clostridia* are important for providing colonization resistance against *C. difficile* due to their ability to produce secondary bile acids as well as potentially competing against *C. difficile* for similar nutrients. This hypothesis was tested by examining the ability of four commensal *Clostridia* encoding the *bai* operon (*C. scindens* VPI 12708, *C. scindens* ATCC 35704, *C. hiranonis* TO 931, and *C. hylemonae* TN 271) to convert CA to DCA *in vitro*. The amount of DCA produced was analyzed and the inhibitory effect of the supernatants against a clinically relevant strain of *C. difficile* was tested. We also investigated the competitive relationship between these commensals and *C. difficile* using an *in vitro* co-culture system. We found that inhibition of *C. difficile* growth by commensal *Clostridia* supplemented with CA was strain-dependent, and correlated with the production of ~2 mM DCA, and increased expression of *bai* operon genes. We also found that *C. difficile* was able to outcompete all four commensal *Clostridia* in an *in vitro* co-culture system. These studies will be instrumental in understanding the relationship between commensal *Clostridia* and the pathogen *C. difficile* in the gut, which is vital for designing targeted bacterial therapeutics. Future studies dissecting the regulation of the *bai* operon *in vitro* and *in vivo* and how this affects CDI will be important.

Methods

Genomic analysis of commensal *Clostridia* strains and the *bai* operon. The *bai* operon alignment was constructed by first extracting the positional information for each *bai* gene of interest from Geneious [41], then obtaining amino acid identity percentage through BLASTp alignments [42] against coding sequences from the reference strain *C. scindens* ATCC 35704. This data was visualized using the publicly available gggenes R package [43] with slight modifications. All vs *C. scindens* ATCC 35704 alignments were visualized using the BLAST Ring Image Generator [44], including entries for GC Content and GC Skew. BLASTn was used for this

alignment, with an upper identity threshold of 90%, a lower identity threshold of 50%, and a ring size of 30.

Bacterial strain collection and growth conditions. The *C. difficile* strain used throughout this study was R20291, a clinically relevant strain from the 027 epidemic ribotype [9]. Additional *C. difficile* strains used were CD196 also from ribotype 027, CF5 and M68 from the 017 ribotype, and 630 from ribotype 012 [9]. All assays using *C. difficile* were started from spore stocks, which were prepared and tested for purity as described previously [9, 45]. *C. difficile* spores were maintained on brain heart infusion (BHI) media supplemented with 100 mg/L L-cysteine and 0.1% taurocholate (T4009, Sigma-Aldrich). Then cultures were started by inoculating a single colony from the plate into BHI liquid media supplemented with 100 mg/L L-cysteine.

Four strains of commensal *Clostridia* encoding the *bai* operon were used in this study. *Clostridium scindens* ATCC 35704 (Cat #35704) was purchased from the American Type Culture Collection. *Clostridium scindens* VPI 12708, *Clostridium hylemonae* TN-271 and *Clostridium hiranonis* TO-931 were obtained from Jason M. Ridlon (University of Illinois Urbana-Champaign, United States). All strains were maintained on 15% glycerol stocks stored in -80 ºC until use and were grown in BHI supplemented with 100 mg/mL L-cysteine. Media for *C. hiranonis* was BHI supplemented with 100 mg/mL L-cysteine and 2 μM hemin. All strains used in this study were grown under 2.5% hydrogen in anaerobic conditions (Coy, USA) at 37 ºC.

Minimum inhibitory concentration (MIC) assay with the addition of bile acids. MICs were determined using the modified Clinical and Laboratory Standards Institute broth microdilution method as described previously [45]. The inoculum was prepared by the direct colony suspension method. All cell concentrations were adjusted to an optical density at 600 nm (OD_{600}) of 0.01. Briefly, the MIC plates were prepared by making fresh bile acid dilution stocks in the test media,

then adding 90 µL to each well such that the final concentration of the bile acid after the addition of cells (10 µL) ranged from 0.04 mM to 10 mM for CA (102897, MP Biomedicals), and 0.01 mM to 2.5 mM for DCA (D6750, Sigma Aldrich). Four biological replicates were performed. Positive controls were inoculated cells with no bile acid in test media. Uninoculated media was used as a control for sterility. *C. scindens, C. hiranonis* and *C hylemonae* were incubated for 48 hr while *C. difficile* was incubated for 24 hr anaerobically at 37 ºC. All assays were performed in BHI supplemented with 100 mg/L of L-cysteine. For assays involving *C. hiranonis,* the BHI was also supplemented with 2 µM hemin.

Growth kinetics assay. Cultures of *C. scindens, C. hiranonis, C. hylemonae* and *C. difficile* liquid cultures were started from a single colony and grown for 14 hr, then sub-cultured 1:10 and 1:5 in liquid media and allowed to grow for 3 hr or until doubling. Cultures were then diluted in fresh BHI supplemented with varying concentrations of CA or DCA so that the starting OD_{600} was 0.01. The growth media for *C. hylemonae* was BHI supplemented with 2 µM hemin and varying concentrations of CA or DCA. Growth studies were performed in 200 µL of media over 24 hr using a Tecan plate reader inside the anaerobic chamber. OD_{600} was measured every 30 min for 24 hr, and the plate was shaken for 90 sec before each reading was taken. Three technical replicates were performed for each concentration of bile acid, and three biological replicates were performed for each organism.

***C. difficile* inhibition assay with supernatants from commensal *Clostridia* supplemented with and without bile acids.** Cultures of *C. scindens* and *C. hiranonis* were grown in fresh BHI supplemented with 0 mM, 0.25 mM or 2.5 mM of CA, while cultures of *C hylemonae* were grown in fresh BHI supplemented with 0 mM, 2.5 mM or 7 mM of CA. After 14 hr of growth, cultures were spun down anaerobically at 6,000 rpm for 5 min. Supernatants were then sterilized under

anaerobic conditions using a 0.22 μM filter and used in the inhibition assay at a ratio of 4 parts supernatant to one part BHI.

Cultures of *C. difficile* were started from a single colony and grown for 14 hr, then subcultured 1:10 and 1:5 in liquid media and allowed to grow for 3 hr or until doubling, then diluted to 0.01 OD in a mixture of 4 parts filtered supernatant to 1 part BHI. *C. difficile* grown in a mixture of 4 parts PBS to 1 part BHI was used as a control for this assay.

Bile acid controls included *C. difficile* grown in BHI supplemented with 0.25 mM, 2.5 mM or 7 mM CA as well as BHI supplemented with 0.25 mM or 2.5 mM DCA. *C. difficile* grown in BHI without bile acid supplementation was used as a positive control. Cultures were allowed to incubate for 24 hr anaerobically at 37 ºC, then dilutions were plated on BHI plates, and the number of colony forming units (CFUs) was calculated the next day. Aliquots of the supernatants from *C. scindens, C. hiranonis* and *C. hylemonae* from this assay were stored at -80 ºC for later bile acid metabolomic analysis.

Bile acid metabolomic analysis. Culture media supernatants were diluted 1:100 in methanol (2 μL supernatant, 198 μL MeOH) and analyzed by UPLC-MS/MS. BHI media alone was also analyzed for the presence of CA and DCA. Both CA and DCA were below the lower limit of quantification (LLOQ) in BHI media controls. The analysis was performed using a Thermo Vanquish LC instrument (Thermo Fisher Scientific, San Jose, CA) coupled to a Thermo TSQ Altis triple quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA) with a heated electrospray ionization (HESI) source. Chromatographic separation was achieved on a Restek Raptor C18 column (2.1 x 50 mm, 1.8 mM) maintained at 50°C. The following linear gradient of mobile phase A (5 mM ammonium acetate) and mobile phase B (1:1 MeOH/MeCN) was used: 0-2 min (35-40%B, 0.5 mL/min), 2-2.5min (40-45%B, 0.5 mL/min), 2.5-3.5 min (45-50%B, 0.5

mL/min), 3.5-4.6 min (50-55%B, 0.5 mL/min), 4.6-5.7 min (55-80%B, 0.8 mL/min), 5.7-5.9 min (80-85%B, 0.8 mL/min), 5.9-6.5 min (85%B, 0.8 mL/min), 6.5-8.5 min (35%B, 0.5 mL/min). For quantification, certified reference material (50 mg/mL) for CA and DCA were obtained from Cerilliant. These individual stocks were combined and diluted to achieve a 250 mM working standard solution. Seven calibration standards ranging from 8 nM to 125 mM were prepared by serially diluting the working standard solution. Both samples and standards were analyzed (2 mL injections) in negative ion mode (spray voltage 2.5 kV, ion transfer tube temperature 325°C, vaporizer temperature 350°C, sheath gas 50 a.u., aux gas 10 a.u., sweep gas 1 a.u.) using a Q1 resolution of 0.7 m/z, a Q3 resolution of 1.2 m/z and a collision energy of 22 V. The following multiple reaction monitoring (MRM) transitions were used: 407.1 →407.1 (CA), 391.1 → 391.1 (DCA).

Table 1: Candidate reference genes

Putative housekeeping gene	Function
rpoC	RNA polymerase subunit C
gyrA	Gyrase subunit A
gluD	Glutamate dehydrogenase
adk	Adenylate kinase
dnaG	DNA primase
recA	Recombination protein A
rpsJ	30S ribosomal protein S10

Targeted data processing. Peak integration and quantification were performed in TraceFinder 4.1 (Thermo Fisher Scientific, San Jose, CA). Individual standard curves for CA and DCA were constructed using peak areas from the quantifier transitions (407.1 → 407.1 for CA, 391.1 → 391.1 for DCA). The concentrations of CA and DCA in the study samples were calculated in an identical manner relative to the regression line. Calibration curves for CA and DCA had R^2 values ranging of 0.9995 and 0.9983, respectively, for the linear range of 8 nM to 125 mM with a weighting of 1/x. Validation of the curve with a QC sample (2.5 mM) passed with a threshold of 10%.

RNA extraction from commensal *Clostridia* cultures supplemented with CA. *C. scindens, C. hiranonis* and *C. hylemonae* liquid cultures were started from a single colony and grown for 14 hr, then subcultured 1:10 and 1:5 in liquid media and allowed to grow for 3 hr or until doubling. Cultures of *C. scindens* and *C. hiranonis* were then diluted to 0.1 OD in fresh BHI supplemented with 0 mM, 0.25 mM or 2.5 mM of CA, while cultures of *C hylemonae* were diluted to 0.1 OD in fresh BHI supplemented with 0 mM, 2.5 mM or 7 mM of CA. Cultures were allowed to grow to mid log phase (OD of 0.3-0.5) then half of the culture was removed and stored for later extraction. The remaining portion of the culture was allowed to grow for 14 hr until stationary phase was reached.

Cultures were fixed by adding equal volumes of a 1:1 mixture of EtOH and acetone and stored in the -80 ºC for later RNA extraction. For extraction, the culture was thawed then centrifuged at 10,000 rpm for 10 min at 4 ºC. The supernatant was discarded and the cell pellet resuspended in 1 mL of 1:100 BME:H_2O, then spun down at 14,000 rpm for 1 min. The cell pellet was resuspended in 0.3 mL of lysis buffer from the Ambion RNA purification kit (AM1912, Invitrogen) then sonicated while on ice for 10 pulses of 2 sec with a pause of 3 sec between each pulse. Extraction was then performed following the manufacturers protocol from the Ambion RNA purification kit.

Reverse transcription and quantitative real-time PCR. Reverse transcription and quantitative real-time PCR was performed as described previously [30]. Briefly, RNA was depleted by using Turbo DNase according to the manufacturer's instructions (AM2238, Invitrogen. The DNased RNA was then cleaned using an RNA clean up kit (R1019, Zymo) according to manufacturer

Table 2: Primer sequences used

Gene	Organism	Primer direction – sequence (5′–3′)
rpoC	*C. scindens*	F – GGACTCTTACCAGTGGTTTCTG
		R – CGTCATCCTCGCACAAAGTA
	C. hylemonae	F – GTGCGAGGATGATGTGAAGTA
		R – CATCGGTTTCCTTGTTGTGAAG
	C. hiranonis	F – ACAGTTCATGGACCAGACTAAC
		R – GAACCCAGCTCTTTCTCTTGA
gyrA	*C. scindens*	F – CCATCTATGGAGCACTGGTAAA
		R – CCATCTACGGAGCCAAAGTT
	C. hylemonae	F – AGACGCTGGAAGAGGACTA
		R – GGTATACGCGGCCTGTATTT
	C. hiranonis	F – GCAGTTGGTATGGCTACATCTAT
		R – CAACATCTGCATCTGGTCTATCT
gluD	*C. scindens*	F – TGCGTTCACGGAGTTGTT
		R – GTACATTCTTGACGGTGAACATAAC
	C. hylemonae	F – TTCACCGGGAAGGAATGAAG
		R – TGGGACAGGTAGTCGAGAAT
	C. hiranonis	F – GGTGCCCTAAACATGGTACTT
		R – AAATCTCCGTTAGCTGCTCTG
adk	*C. scindens*	F – ATTGCCGCAAAGTATGGTATTC
		R – TCCATGTACGTCTTGGCTTT
	C. hylemonae	F – ACGTTGATGTTCCGGATGAG
		R – GTCACAGATGCCGTCTTTCT
	C. hiranonis	F – AGTAGGAAGAGCTGTAGGAAGA
		R – TCGTCTGCTCTCTGATGTAGA
dnaG	*C. scindens*	F – GGAAACTTTGGCTCCGTAGAT
		R – GTTGATATCAGCAGTCAGTTCCA
	C. hylemonae	F – GCAGGCAGAAGCAGATGTA
		R – AGCGCTTCCAGAAACGTATAG
	C. hiranonis	F – GATGCTGTACCTCGGATTAAGT
		R – ACAGGGCTCTTTAGCTGTTT
recA	*C. scindens*	F – GAGACGATTCCCACAGGTTC
		R – CTGGATTCCGGGCCATATAC
	C. hylemonae	F – GCGCGATGGATATCGTAGTT
		R – CTAAGCGCCTGGGACATAAG
	C. hiranonis	F – AGGAGAACAGGCACTAGAGATA
		R – TCACCTTCTATTTCGGCTTTAGG
rpsJ	*C. scindens*	F – GAGAATCACTTTGAAAGCGTATGA
		R – GCTCACCTGTGATCCATTCT
	C. hylemonae	F – GGTCGCTCTCGTTGAAGAAT
		R – GCCTCCACAACTGGAATGATA
	C. hiranonis	F – GACCTTAAAGAAGCAGTAGTAGGT
		R – TCCAGTGAAAGCTCCGTTATC
baiG	*C. scindens* ATCC 35704	F – GGTAACTTCCTTCGGGCTATG
		R – GGATGGTCTTCCACAGCATTTA
	C. scindens VPI 12708	F – CCCATCCTTCGGACAGAATAT
		R – GATCGGACTCTTCGCTTTCTT
	C. hylemonae	F – GGCCGTATTCCTGATCTGTATG
		R – CACAGGAACAGGCTGAAGAA
	C. hiranonis	F – ATGATCGGACTCTTCGCTTTC
		R – AGCTTCTGCAGTTCCGTTAG
baiE	*C. scindens* ATCC 35704	F – GGTAACTTCCTTCGGGCTATG
		R – GACGGAAAGATGTGGGATGAA
	C. scindens VPI 12708	F – GACGGAAAGATGTGGGATGAG
		R – CTTCCTTCGGGCTATGGAATAC
	C. hylemonae	F – CCCGAACATTGTAACTTCCTACT
		R – GTCCCATGTGCATGCTTATTTC
	C. hiranonis	F – CTGCTACAGGAAGATGGTACTT
		R – GTAGAATGCTCCACCGTTTATTC
baiN	*C. scindens* ATCC 35704	F – GTTCCAGCAGTCTTGGGATTA
		R – CAGGTACGCAAGGCATTTAAC
	C. scindens VPI 12708	F – TGACACGCTGGAAGAAGTAATC
		R – CGTAATCAAAGGTCCCATCCTC
	C. hylemonae	F – GGATGTGATCTGCATGGTAGAC
		R – CCGGGATTCTTTATGAGGGAAC
	C. hiranonis	F – AGG GTA CGA AGA CGA GGT TAT
		R – GTAAGTCCACCCTGGAAGTAAAC

instruction and DNA depletion was verified by amplifying 1 µL of RNA in a PCR reaction. The DNA depleted RNA was used as the template for reverse transcription performed with Moloney murine leukemia virus (MMLV) reverse transcriptase (M0253, NEB). The cDNA samples were then diluted 1:4 in water and used in quantitative real-time PCR with gene-specific primers using SsoAdvanced Universal Sybr green Supermix (1725271, Bio-Rad) according to the manufacturer's protocol. Amplifications were performed in technical quadruplicate, and copy numbers were calculated by the use of a standard curve and normalized to that of a housekeeping gene. *gyrA* was the housekeeping gene used for *C. scindens* and *rpoC* was the housekeeping gene used for *C. hiranonis* and *C. hylemonae.*

The housekeeping gene for each strain was determined by testing a list of genes (Table 1 and Table 2) using cDNA standardized to a concentration of 0.3 ug/µL. Three technical replicates were performed for each assay, and three biological replicates were performed. *C. scindens* and *C. hiranonis* were tested with RNA from cultures grown to mid log and stationary phase in media supplemented with 0 mM, 0.25 mM or 2.5 mM of CA, while *C. hylemonae* was tested with RNA from cultures grown to mid log and stationary in media supplemented with 0 mM, 2.5 mM or 7 mM of CA, and copy numbers were calculated by the use of a standard curve. Analysis of copy numbers was performed with Normfinder [46] and the gene with the lowest inter-group variance, or stability value was selected (Table 3). The Log_2 fold change for each gene and each condition (Table S1) was calculated by dividing the expression when CA was added by the expression of the negative control (0 mM CA).

Competition studies between *C. difficile* and commensal *Clostridia*. *C. scindens, C. hylemonae* and *C difficile* liquid cultures were started from a single colony as described above. Monocultures of each organism were diluted to ~$1x10^5$ CFU/mL in fresh BHI media, and 1:1 competition assays

Table 3: NormFinder analysis results

Strain	Gene	Stability value
C. scindens ATCC 35704	***gyrA***	0.41
	recA	0.45
	rpoC	0.50
	dnaG	0.59
	adk	0.94
	rpsJ	0.99
	gluD	2.04
C. scindens VPI 12708	***gyrA***	0.70
	recA	0.73
	dnaG	0.84
	rpoC	1.10
	adk	1.40
	rpsJ	1.55
C. hylemonae	***rpoC***	0.63
	gyrA	0.65
	dnaG	0.89
	recA	1.16
	gluD	1.21
	adk	1.54
	rpsJ	1.79
C. hiranonis	***rpoC***	0.34
	dnaG	0.38
	gyrA	0.44
	adk	0.47
	gluD	0.48
	recA	0.52
	rpsJ	0.56

were ~1x10^5 CFU/mL of each organism in fresh media. Determination of the conversion from OD to CFU/mL was performed for *C. difficile, C. scindens* and *C. hylemonae* using a growth curve to measure OD and CFU/mL. The conversion from OD to CFU/mL was calculated using a linear regression analysis (Figure S1).

Dilutions were performed at 0 hr and after 24 hr of incubation, and colonies were counted to determine the number of CFUs per mL. Colonies were counted after the plates had been incubated long enough to allow individual colonies to form, which was 24 hr for *C. difficile* and 48 hr for *C. scindens* and *C. hylemonae*. Differences in colony morphology and growth time were

used to distinguish between *C. scindens*, *C. hylemonae* and *C. difficile.*

Statistical analyses. Statistical tests were performed using Prism version 7.0c for Mac OS X (GraphPad Software, La Jolla, CA, United States). Significance was determined by using one-way ANOVA to determine significance across all conditions, with Tukey's test used to correct for multiple comparisons. Statistical significance was set at a p value of < 0.05 for all analyses, (*$p < 0.05$, **$p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). All assays were performed with at least three biological replicates.

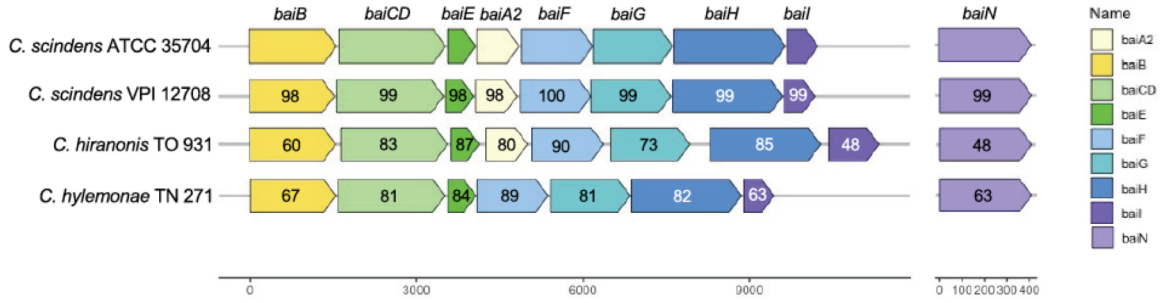

Figure 1: Genomic variation in selected *bai* genes encoded by commensal *Clostridia*. Alignment of the *bai* operon and *baiN* across *Clostridium* strains. Each protein sequence was compared against its counterpart in the reference strain *Clostridium scindens* ATCC 35704, generating the amino acid percent identity labeled within each gene.

Results

Genomic comparison between commensal *Clostridia* that encode the *bai* operon.

The *bai* operons and the *baiN* genes from *C. scindens* VPI 12708, *C. hiranonis* TO 931 and *C. hylemonae* TN 271 were visually aligned and the amino acid sequences were compared to the reference strain *C. scindens* ATCC 35704 (Figure 1). The operons of both *C. scindens* ATCC 35704 and *C. scindens* VPI 12708 are architecturally similar with each gene within the operon sharing at least 97% identity at the amino acid level. Interestingly, *C. hylemonae* TN 271 possesses

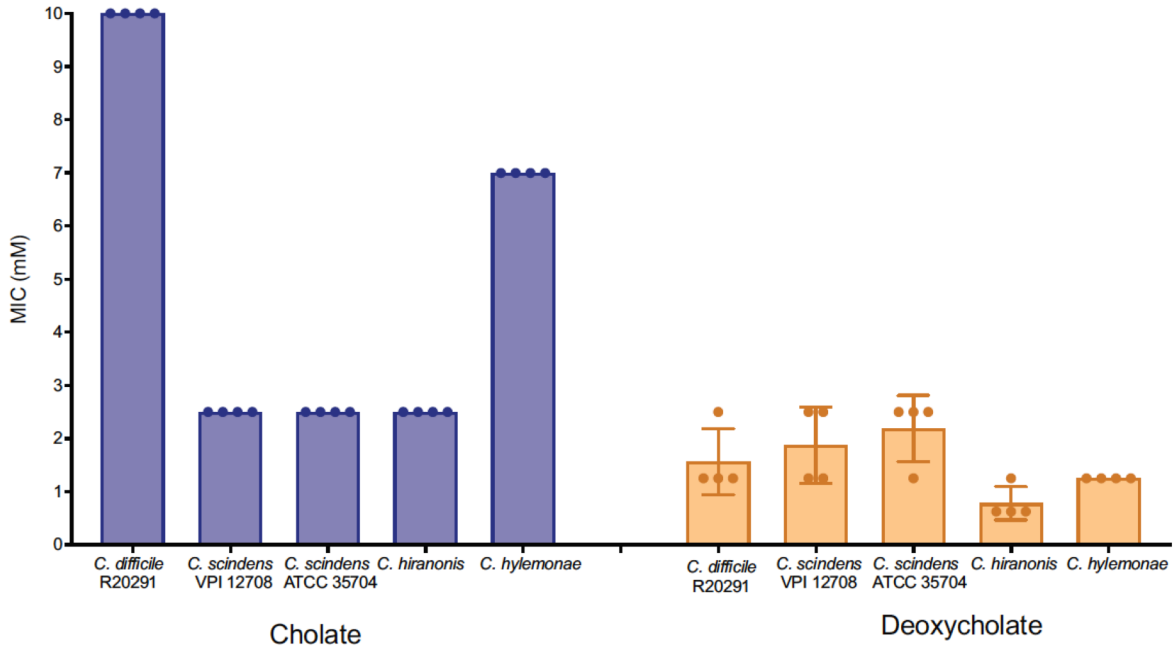

Figure 2: *C. difficile* and *C. hylemonae* are more resistant to cholate than other commensal *Clostridia* tested. The Minimum Inhibitory Concentration (MIC) of CA and DCA was tested on *C. difficile* R20291, *C. scindens* VPI 12708, *C scindens* ATCC 35704, *C. hiranonis* and *C. hylemonae.* The MIC was defined as the lowest concentration of compound that showed no visible growth. Growth was defined at 24 hr for *C. difficile* and 48 hr for commensal *Clostridia.* Four biological replicates were performed.

a shorter *bai* operon due to the lack of the *baiA2* gene, while the *bai* operon of *C. hiranonis* TO 931 is ~ 1 kb longer than the reference operon due to expanded intergenic regions. *C. hylemonae* TN 271 shares between 63% and 89% identity with the reference operon and *C. hiranonis* displays between 48% and 90% identity. Notably, the outermost protein coding sequences in the *bai* operon of *C. hylemonae* TN 271 and *C. hiranonis* TO 931, *baiB* and *baiI*, exhibit a significantly reduced percent identity when compared to other genes in the operon.

The differences in identity across these four operons is largely representative of the whole-genome nucleotide comparison of each strain (Figure S2), with *C. hiranonis* TO931 sharing the least identity to the reference strain *C. scindens* ATCC 35704, followed by the slightly higher percent nucleotide identity of *C. hylemonae* TN271, with *C. scindens* VPI 12708 sharing the most

nucleotide identity.

***C. difficile* is more resistant to cholate and less resistant to deoxycholate than commensal *Clostridia*.**

Since secondary bile acids such as DCA are made by specific commensal *Clostridia* encoding the *bai* operon from CA and are inhibitory against *C. difficile,* we sought to examine the resistance profiles of these bacteria to CA and DCA [9]. We did this by performing minimum inhibitory concentration (MIC) assays with CA and DCA for multiple strains of *C. difficile* as well as four commensal *Clostridia* that encode the *bai* operon [14-16]. *C. difficile* R20291 had a high MIC of 10 mM with CA, but a much lower MIC of 1.56 mM with DCA (Figure 2, Table S1). When four other *C. difficile* strains were tested, the results were similar, with 630, CD196, CF5 and M68 all having MICs greater than 10 mM for CA and MICs of 1.25 mM for DCA (Figure S3). Of the commensal *Clostridia, C. hylemonae* had the highest MIC against CA with 7 mM, while *C. hiranonis* and the two *C. scindens* strains all had a MIC of 2.5 mM for CA. Of the commensals, *C. hiranonis* was most sensitive to DCA, with a MIC of 0.78 mM. *C. hylemonae* was also sensitive to DCA, with a MIC of 1.25 mM. The two *C. scindens* strains were more resistant to DCA, with *C. scindens* VPI 12708 having a MIC of 1.88 mM and *C. scindens* ATCC 35704 having a MIC of 2.19 mM. Although the commensal *Clostridia* tested have all shown the ability to produce DCA *in vitro*, they all display sensitivity to it. Out of the four commensal strains, only the two *C. scindens* strains had higher resistance to DCA than *C. difficile* R20291. Different concentrations of CA and DCA were also able to alter the growth kinetics of the commensal *Clostridia* and *C. difficile* R20291 as seen in Figures S4 and S5.

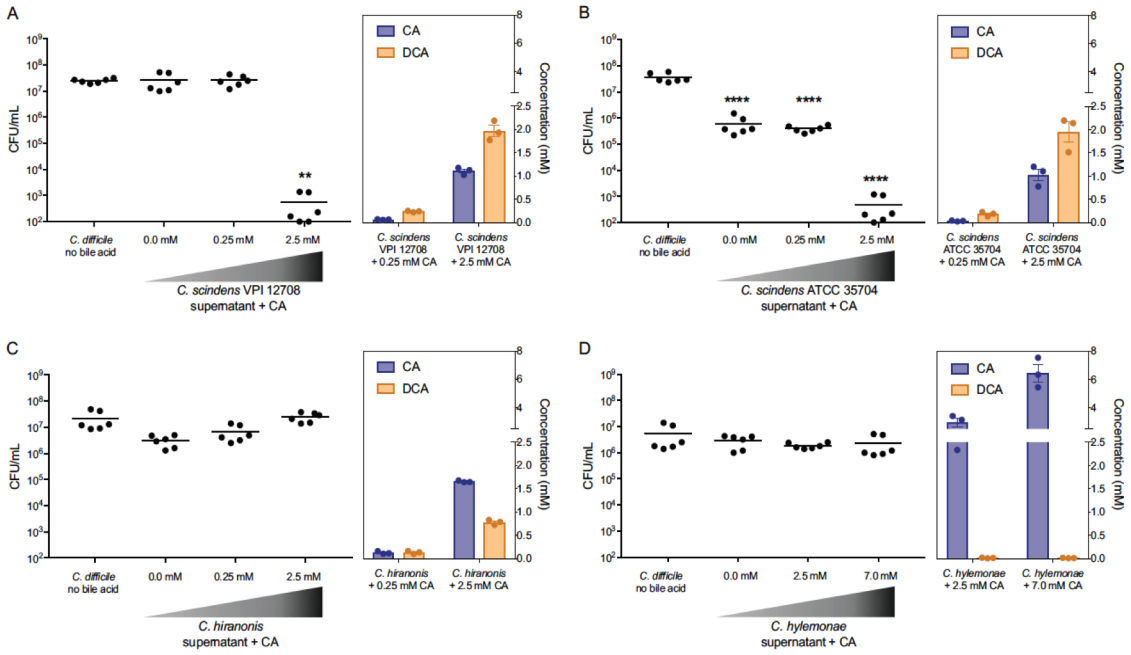

Figure 3: Inhibition of *C. difficile* by *C. scindens* grown in media supplemented with 2.5 mM cholate is correlated with high levels of deoxycholate production. Inhibition of *C. difficile* after 24 hr of growth with supernatants from **(A)** *C. scindens* VPI 12708, **(B)** *C. scindens* ATCC 35704, and **(C)** *C. hiranonis* grown without bile acid supplementation or supplemented with 0.25 or 2.5 mM of CA, and **(D)** *C. hylemonae* grown without bile acid supplementation or supplemented with 2.5 mM or 7.0 mM of CA. The concentration of CA and DCA in each supernatant is shown to the right of the inhibition data. Experiments were run in duplicate, and three biological replicates were performed. Inhibition by the supernatants was compared to a no bile acid *C. difficile* control consisting of a 4:1 dilution of PBS to BHI. Statistical significance between treatments and the control was determined using one-way ANOVA with Tukey used for multiple comparisons (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$).

Commensal *Clostridia* inhibit *C. difficile* in a strain-dependent manner that correlates with the conversion of cholate to deoxycholate.

While all four commensal *Clostridia* examined in this study have been shown to produce DCA from CA *in vitro*, we wanted to ascertain whether this was sufficient to inhibit *C. difficile* growth [15, 16, 47]. We developed an *in vitro* inhibition assay using supernatants from overnight cultures of commensal *Clostridia* supplemented with and without different concentrations of CA. The

supernatants were then added to fresh *C. difficile* cultures to investigate if commensal *Clostridia* capable of producing DCA from CA were able to inhibit *C. difficile* growth. When the supernatant from *C. scindens* VPI 12708 cultures supplemented with 0.25 mM of CA and no CA were added to *C. difficile* cultures, there was no inhibition of growth after 24 hr. When *C. scindens* VPI 12708 cultures were supplemented with 2.5 mM of CA, the supernatant significantly inhibited *C. difficile* growth (Figure 3A). The level of inhibition was similar to the level of inhibition seen when *C. difficile* was grown with 2.5 mM of DCA alone (Figure S6). In order to determine the levels of CA and DCA present in the supernatants added to *C. difficile* cultures, targeted liquid chromatography-mass spectrometry (LC/MS) was performed. *C. scindens* VPI 12708 was able to convert almost all of the CA present in the media to DCA (Figure 3A). When the media was supplemented with 0.25 mM of CA, 0.24 ± 0.02 mM DCA was produced, and 1.97 ± 0.21 mM DCA was produced when the media was supplemented with 2.5 mM CA. Supernatant from *C. scindens* ATCC 35704 cultures also greatly inhibited *C. difficile* when grown in media supplemented with 2.5 mM of CA (Figure 3B), but some inhibition was also seen with supernatant from cultures supplemented with 0.25 mM CA and no CA. This is likely due to the production of tryptophan-derived antimicrobials produced by this *C. scindens* strain that have previously been shown to inhibit *C. difficile* [48]. *C. scindens* ATCC 35704 also converted most of the CA present in the media to DCA (Figure 3B) with 0.19 ± 0.05 mM of DCA being produced when the media was supplemented with 0.25 mM CA, and 1.95 ± 0.38 mM DCA being produced when supplemented with 2.5 mM CA. *C. hiranonis* and *C. hylemonae* culture supernatants did not significantly inhibit *C. difficile* growth (Figure 3C and D), regardless of the amount of CA present in the culture media. *C. hiranonis* did convert some of the CA to DCA (Figure 3C), with 0.14 ± 0.03 mM DCA being produced when supplemented with 0.25 mM CA, and 0.78 ± 0.05 mM DCA produced when supplemented with

2.5 mM CA. *C. hylemonae* did not convert any of the CA present in the media to DCA (Figure 3D).

Expression of *baiE* and *baiG* is increased when cholate is converted to deoxycholate.

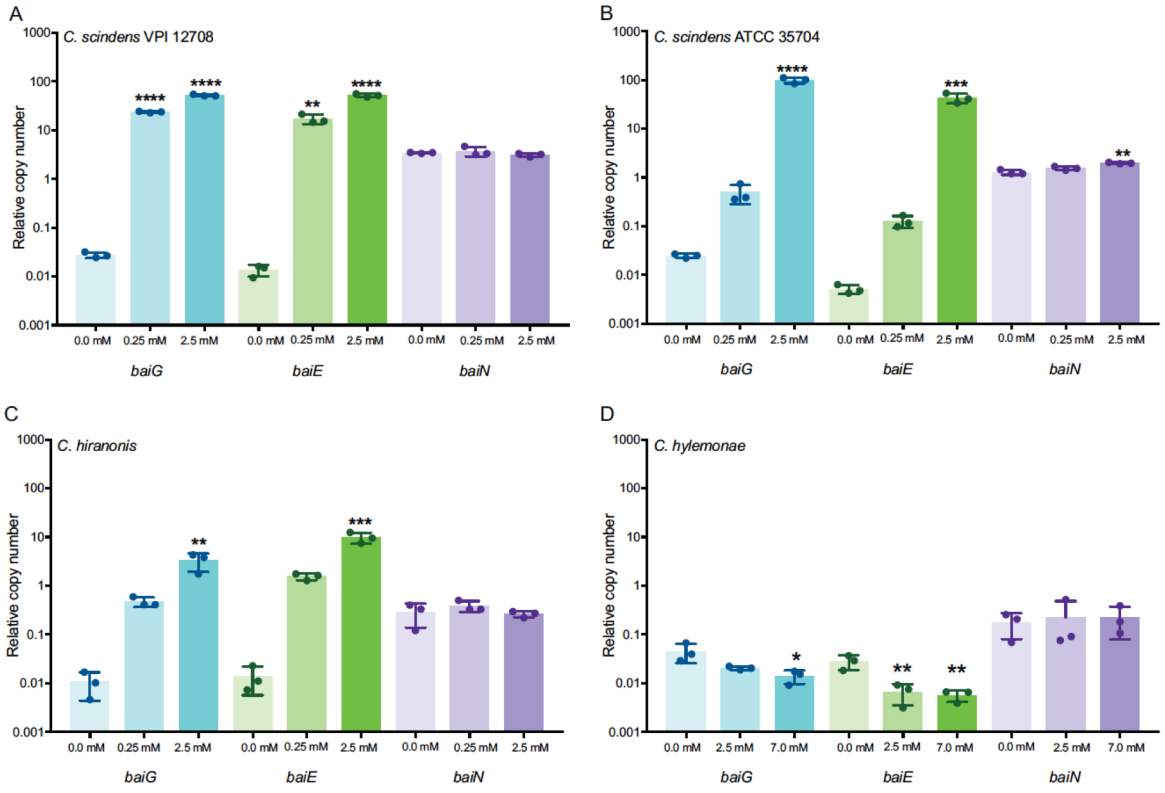

Figure 4: *C. scindens* and *C. hiranonis* have increased expression in *bai* operon genes when media is supplemented with 2.5 mM CA. Expression of *baiG, baiE* and *baiN* in **(A)** *C. scindens* ATCC 35704, **(B)** *C. scindens* VPI 12708, and **(C)** *C. hiranonis* in media without CA or media supplemented with 0.25 mM or 2.5 mM CA, and **(D)** *C. hylemonae* in media without CA or media supplemented with 2.5 mM or 7.0 mM CA. Experiments were run in quadruplicate, and three biological replicates were performed. Expression in media supplemented with CA was compared to expression in media without CA. Statistical significance was determined by one-way ANOVA with Tukey used for multiple comparisons (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$).

To investigate the effect of different concentrations of CA on expression of the *bai* operon, three genes were selected for qRT-PCR analysis. We selected *baiG,* which is responsible for transport of CA into the cell, *baiE,* which is responsible for the irreversible and rate limiting conversion from 3-oxo-4,5-dehydro-cholyl-CoA to 3-oxo-4,5-6,7-didehydro-deoxy-cholyl-CoA, and *baiN,*

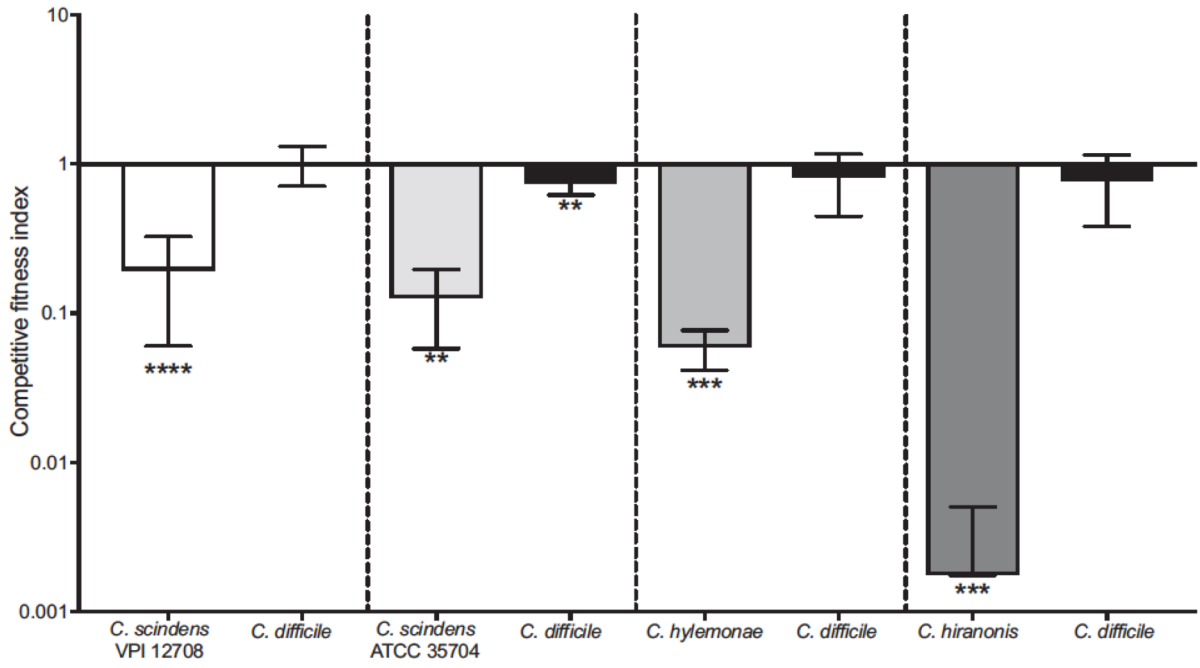

Figure 5: *C. difficile* outcompetes commensal *Clostridia in vitro*. Competition index for 1:1 competition between *C. difficile* and *C. scindens* ATCC 35704, *C. scindens* VPI 12708, *C. hylemonae* and *C. hiranonis.* The competition index value was determined by comparing the CFU/mL of the competition co-culture to the monoculture for each strain at 24 hr. Statistical significance was determined by using students T test (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$).

which is capable of performing the first two reductive steps in the pathway [18, 19, 49]. Commensal *Clostridia* cultures were grown in a rich medium supplemented with different concentrations of CA for a 24 hr period, followed by RNA extraction before qRT-PCR analysis. *C. scindens* VPI 12708 had a significant increase in expression of *baiE* and *baiG* when 0.25 mM or 2.5 mM of CA was present in cultures (Figure 4A). *C. scindens* ATCC 35704 also showed a significant increase in expression in *baiE* and *baiG* when 2.5 mM of CA was present in cultures, but not with 0.25 mM of CA (Figure 4B). *C. hiranonis* had a significant increase in expression in *baiE* and *baiG* when 2.5 mM of CA was present in cultures (Figure 4C), but the increased expression is approximately ten fold less than either *C. scindens* strain with an equal amount of CA in the media. *C. hylemonae* had a decrease in expression of *baiE* when 2.5 mM CA was added (Figure 4D), and decreased

expression of *baiE* and *baiG* when 7 mM of CA was added. Expression of *baiN* was not affected by the different CA concentrations in the media, although a small but significant increase was seen in *C. scindens* ATCC 35704 when supplemented with 2.5 mM CA.

***C. difficile* outcompetes commensal *Clostridia* in a strain dependent manner.**

In order to explore our additional hypothesis that commensal *Clostridia* are able to compete against *C. difficile* for similar nutrients, we performed 1:1 competition assays between commensal *Clostridia* and *C. difficile* in rich media without the addition of CA (Figure 5 and Figure S7). A monoculture of each strain was used as a control for each replicate performed. The competition index (Figure 5) was calculated by dividing the CFU of each strain after 24 hr of growth in the 1:1 competition assay with the CFU of the monoculture control after 24 hr. The raw data from the competition assays is available in Figure S7. All commensal *Clostridia* growth was significantly inhibited in co-culture with *C. difficile,* with *C. hiranonis* being affected the most. *C. difficile* growth was not negatively affected by any commensal strain except for *C. scindens* ATCC 35704, which is consistent with the inhibition observed by *C. scindens* ATCC 35704 culture supernatant without CA in Figure 3A and the inhibition observed by Kang et al. [48].

Discussion

In this study we examined the genomic variation between four commensal *Clostridia* strains containing the *bai* operon, determined their ability to produce DCA when supplemented with CA, and investigated their ability to inhibit *C. difficile in vitro*. While *C. scindens* VPI 12708, *C. scindens* ATCC 35704 and *C. hiranonis* all produced DCA under these conditions (Figure 4), only *C. scindens* strains were able to inhibit *C. difficile* when supplemented with 2.5 mM CA. This is likely due to the efficient conversion of CA to DCA produced when they were supplemented with 2.5 mM CA. *C. hiranonis* produced less DCA (0.78 mM) when supplemented with 2.5 mM CA.

This could be due to the lower MIC of *C. hiranonis* with DCA (0.78 mM) compared to *C. scindens* VPI 12708 (1.88 mM) and *C. scindens* ATCC 35704 (2.19 mM). Additionally, *C. difficile* inhibited the growth of all four commensals tested in a 1:1 *in vitro* competition assay without the presence of CA, with only *C. scindens* ATCC 35704 affecting *C. difficile* growth, which is likely due to the antimicrobial activity it produces [48].

The proteins encoded by the *bai* operon of *C. scindens* VPI 12708 had a high amino acid similarity (~97%) to those of *C. scindens* ATCC 35704, but *C. hylemonae* and *C. hiranonis* were more divergent than expected. This pattern continued when examining the genome, with *C. hiranonis* and *C. hylemonae* diverging from *C. scindens* ATCC 35704 more than *C. scindens* VPI 12708. Of particular interest is the lack of *baiA2* in the *bai* operon in *C. hylemonae. BaiA2* encodes a short chain dehydrogenase/reductase that is responsible for two steps in the 7α-dehydroxylation pathway. One of those steps is the conversion from cholyl-CoA to 3-oxo-cholyl-CoA in the oxidative arm, and the other is the conversion from 3-oxo-DCA to DCA in the reductive arm of the pathway [13, 50]. However, it is important to note that while *C. hylemonae* lacks *baiA2* in the main *bai* operon*, baiA1* is present elsewhere in the genome under the control of a different promoter [12]. *BaiA1* can also perform the conversion from cholyl-CoA to 3-oxo-cholyl-CoA [50, 51]. It is also important to note that there may be other redundancies built in to the 7α-dehydroxylation pathway. When *baiN* was expressed in *E. coli*, it was capable of performing two conversions, that of 3-oxo-4,5-6,7-didehydro-DCA to 3-oxo-4,5-dehydro-DCA then to 3-oxo-DCA. More recently, the entire *bai* operon pathway was expressed in *C. sporogenes* and showed the same two steps being performed by different enzymes, BaiH and BaiCD respectively [13, 49]. While both sets of enzymes are capable of performing these transformations, it is still unknown which one is preferentially utilized by *Clostridia* encoding the *bai* operon.

Given the complexity of the 7α-dehydroxylation pathway and the apparent redundancies built in, the production of DCA is likely very important for the commensal *Clostridia* encoding the *bai* operon. It was surprising to see relatively low MICs of all four commensal *Clostridia* against DCA (Figure 2), as this indicates that the organisms are producing something that is detrimental to them in a sufficient concentration. The reason these commensals produce DCA isn't known, but dietary DCA has been shown to affect the microbiota in chickens and dietary CA supplementation in rats resulted in the outgrowth of *Clostridia* and an increase in DCA, suggesting the production of DCA may modulate the microbiome in a way favorable to these *Clostridia* [52, 53]. While DCA, like other bile acids, has detergent-like properties, the specific mechanism of action of DCA or other bile acids involved in 7α-dehydroxylation, such as CDCA and LCA, against commensal *Clostridia* has yet to be elucidated. DCA and other bile acids have various effects on other bacteria found in the gut. In *Lactobacillus* and *Bifidobacteria,* DCA, CA and CDCA can inhibit growth by dissipating transmembrane electric potential and the transmembrane proton gradient [54]. Bile acids, including DCA, can induce transcription of several genes responsible for DNA repair and recombination in *Escherichia coli, Salmonella typhimurium, Bacillus cereus* and *Listeria monocytogenes* [55]. Genes responsible for maintaining the integrity of the cellular envelope were also upregulated in *B. cereus* and *L. monocytogenes,* indicating that bile acids such as DCA and CDCA damage the bacterial membrane and cellular DNA [55].

Bile acids have multiple effects on *C. difficile* as well. The presence of flagella, as well as the presence of the flagellar structural protein FliC, was significantly decreased when *C difficile* was challenged with DCA, CDCA and LCA, with LCA causing a near complete loss of flagellar filaments [24]. CA did not have any significant effect on flagella in *C. difficile,* but cells challenged with CA, DCA or CDCA were significantly longer than the control cells, while LCA had no

significant effect on cellular shape [24]. While this indicates a potential mechanism for the inhibition of growth by DCA, CDCA and LCA, the mechanism for how these bile acids inhibit *C. difficile* toxin activity is still unknown [9, 24, 56].

C. difficile showed a very high tolerance to CA, with an MIC of 10 mM when grown in 100 µL of BHI (Figure 2). Interestingly, when *C. difficile* was grown in 200 µL of BHI in the growth kinetics assay, growth was observed at 10 mM CA, and complete inhibition only occurred at a concentration of 13 mM CA (Figure S4). It is important to note that while no precipitation was visible in either the MIC or growth kinetics assay, bile acids do form micelles at millimolar concentrations such as the ones used in this study, especially in lipid rich environments such as BHI media, meaning that some of the CA may be in micelles and not interacting with *C. difficile* [57]. CA also appeared to decrease the lag time of *C. difficile* when growth kinetics were analyzed (Figure S4), indicating that supplementation with CA could be beneficial to *C. difficile* growth as well as spore germination [22].

While all four commensal strains tested are capable of making DCA, only the *C. scindens* strains were capable of making enough to inhibit *C. difficile* (Figure 3A-B) in the *in vitro* assay performed. This is likely due to the higher amount of DCA produced when *C. scindens* was supplemented with 2.5 mM CA. Under the conditions tested, *C. hylemonae* did not produce DCA regardless of the amount of CA added to the media (Figure 3D) and no increased activity in the *bai* operon was observed when the media was supplemented with 2.5 mM CA or 7 mM CA. In another study, *C. hylemonae* did have increased expression of several genes in the *bai* operon, including *baiE* and *baiG* in a defined media supplemented with 0.1 mM CA. We tested expression of *baiG, baiE* and *baiN* in BHI media supplemented with 0.1 mM CA, and found no increase in expression (data not shown) [20]. This indicates that regulation of the *bai* operon may be dependent

upon changes in nutritional needs of the bacterium as well as the presence or absence of bile acids, and there may be strain specific differences in regulation. While *C. hiranonis* did produce DCA and had increased expression in *bai* operon genes (Figure 4C) under the conditions tested, supernatants from *C. hiranonis* were not able to inhibit *C. difficile* as it produced less DCA than either *C. scindens* strain (Figure 3C). This is likely due to the susceptibility of *C. hiranonis* to DCA, as it's MIC for DCA was lower than that of *C. difficile* and the amount of DCA produced (0.78 mM) was the same as the MIC for DCA (Table S1). While we did not perform the inhibition assay with CDCA, which is converted to LCA by the *bai* operon, the expression of *baiG, baiE* and *baiN* was tested in *C. scindens* VPI 12708 and *C. hylemonae* cultures supplemented with 0.025, 0.25, and 1.25 mM of CDCA. No significant changes in expression were found in the presence of CDCA when compared to the no bile acid control (data not shown).

While the production of DCA is a factor in the inhibition of *C. difficile* by commensal *Clostridia* containing the *bai* operon, competition for nutrients may also play a role. Like most *Clostridia, C. scindens, C. hylemonae* and *C. hiranonis* encode enzymes that are used in Stickland fermentation, which is required for growth of *C. difficile* and several other *Clostridia* [20, 21, 36, 37]. In particular, proline, which *C. difficile* is auxotrophic for, is one of the most efficient electron acceptors. Another amino acid that most commensal *Clostridia* can utilize for growth is hydroxyproline which can be converted to proline using the *hypD* gene, which is present in many *Clostridia* [58, 59]. There is also the production of other inhibitory metabolites to consider, such as the tryptophan derived antibiotics 1-acetyl-b-carboline and turbomycin A that are produced by *C. scindens* ATCC 35704 and inhibit *C. difficile* [48]. *C. difficile* is also capable of the production of inhibitory molecules. *C. difficle* ATCC 9689 produces proline-based cyclic dipeptides, which inhibit *C. scindens* ATCC 35704, *C. sordellii* and several other bacterial strains commonly found

in the gut [48]. As well as suggesting an important role for tryptophan, which is required for the growth of *C. difficile* and *C. scindens* ATCC 35704, this indicates a potential mechanism for the inhibition of commensal *Clostridia* when co-cultured with *C. difficile* (Figure 5) [21, 35].

Finally, there are some limitations to this study. As was seen with the expression of *baiE* and *baiG* in *C. hylemonae* (Figure 4), the type of media that is used for *in vitro* assays could affect expression of some genes. In addition, *in vitro* assays do not systematically mimic the *in vivo* environment, especially when studying a complex gut environment. In addition, due to the lack of genetic tools available for these commensal *Clostridia* at the time of the study, all assays were performed with wild type strains. This means that in the experiments assessing inhibition of commensal *Clostridia* by CA, the MIC values for *C. scindens* and *C. hiranonis* were likely affected by the conversion of some of the CA in the media to DCA in the assay. While 7α-dehydroxylation also transforms CDCA to LCA, only the inhibition of *C. difficile* by commensal *Clostridia* supplemented with CA was examined in this study due to solubility issues with LCA in media. The genetic intractability of commensal *Clostridia* has made separating the inhibitory effects of 7α-dehydroxylation from other potentially inhibitory mechanisms such as nutrient competition or the production of antimicrobials difficult [48]. However, a recently published CRISPR-Cas9-based method for constructing multiple marker-less deletions in commensal *Clostridia* shows great promise in assisting with this analysis [60]. Future *in vitro* studies using defined media are also needed to further examine the role nutrient competition between *C. difficile* and commensal *Clostridia*. Additional *in vivo* studies are also needed to elucidate the direct mechanistic role 7α-dehydroxylation by commensal *Clostridia* plays in colonization resistance against *C. difficile.* Additional studies dissecting the regulation of the *bai* operon *in vitro* and *in vivo* and how this affects CDI will be important for future therapeutic interventions.

Acknowledgements.

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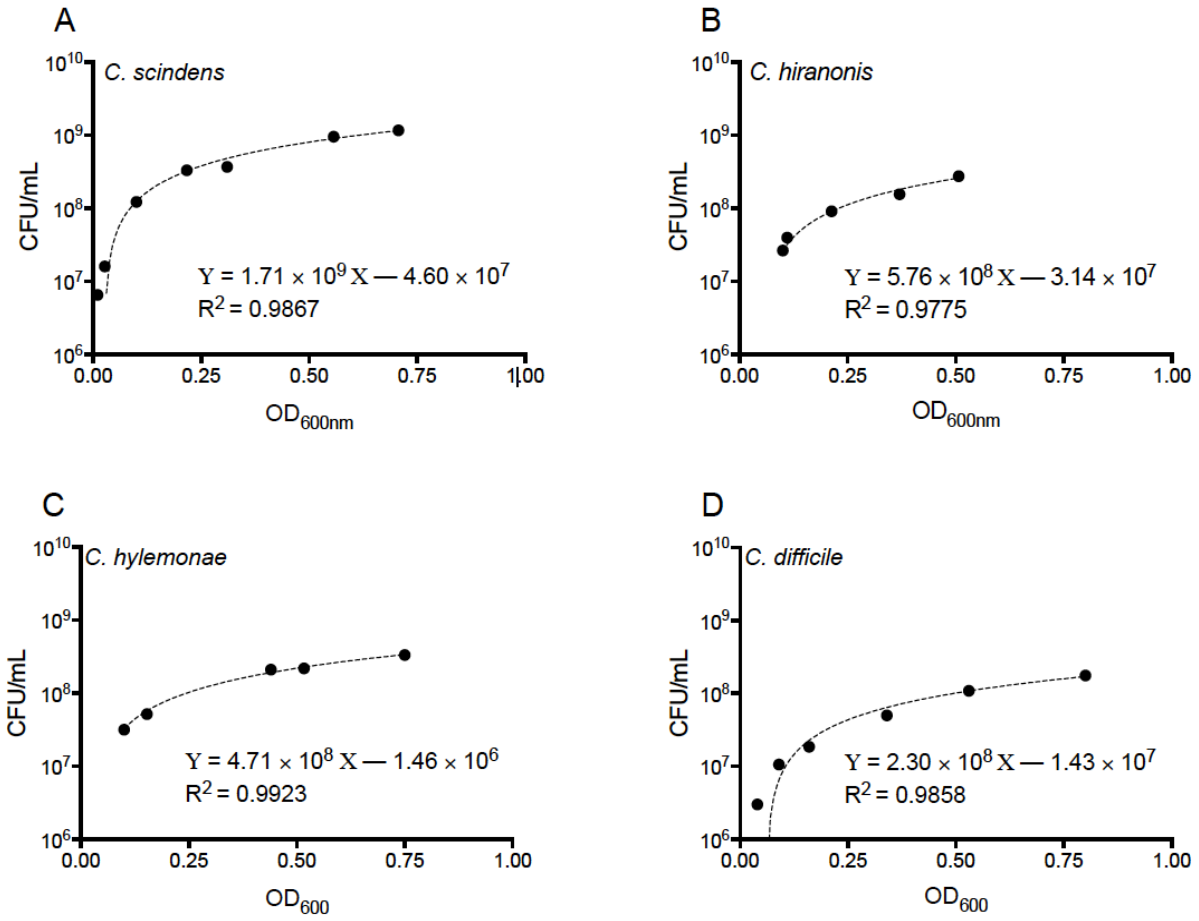
Supplemental Data.

Supplemental Table 1: Log_2 Fold Change for qRT-PCR with addition of CA

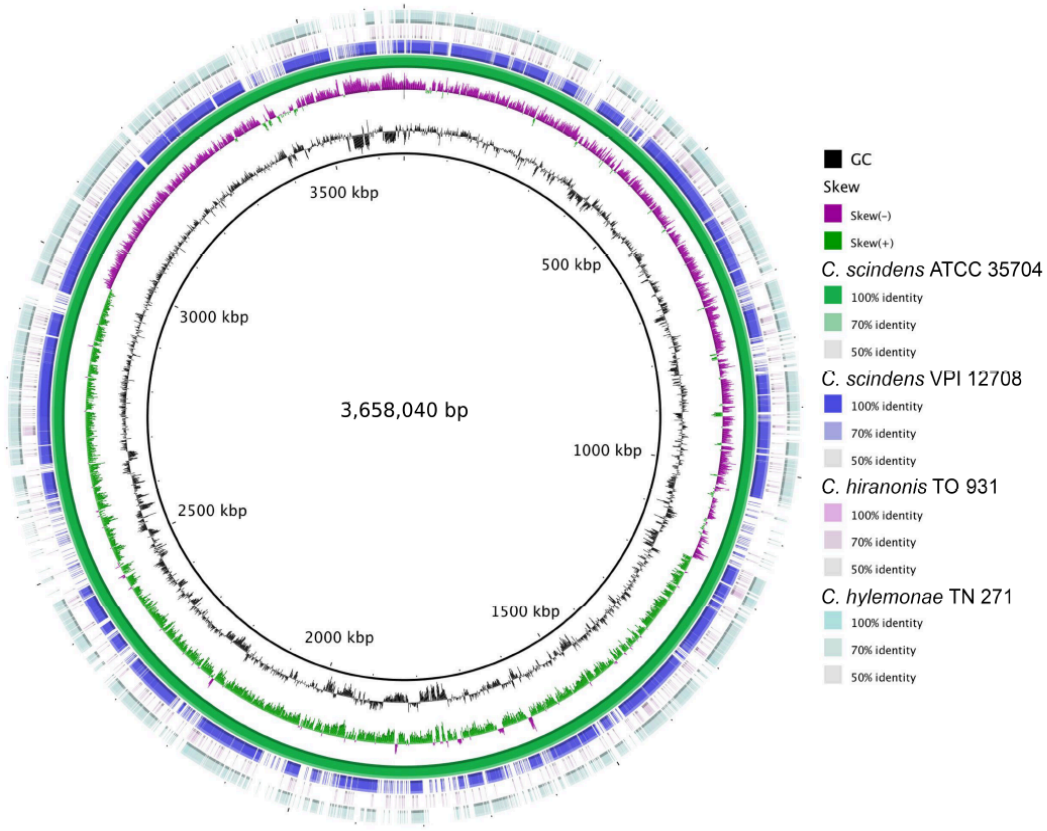
		0.25 mM CA		2.5 mM CA		7.0 mM CA	
Organism	Gene	Avg	SD	Avg	SD	Avg	SD
C. scindens VPI 12708	*baiG*	9.75	0.15	10.89	0.21		
	baiE	10.32	0.72	11.94	0.53		
	baiN	0.10	0.29	-0.15	0.15		
C. scindens ATCC 35704	*baiG*	4.24	0.49	11.96	0.23		
	baiE	4.61	0.60	13.02	0.56		
	baiN	0.37	0.28	0.63	0.20		
C. hiranonis	*baiG*	5.64	0.96	8.36	0.33		
	baiE	6.97	0.88	9.61	0.66		
	baiN	0.58	0.86	0.05	0.74		
C. hylemonae	*baiG*			-1.05	0.74	-1.65	0.28
	baiE			-2.16	1.11	-2.28	0.73
	baiN			-0.01	1.11	0.35	0.45

Supplemental Table 2: Minimum Inhibitory Concentration for CA and DCA (mM)

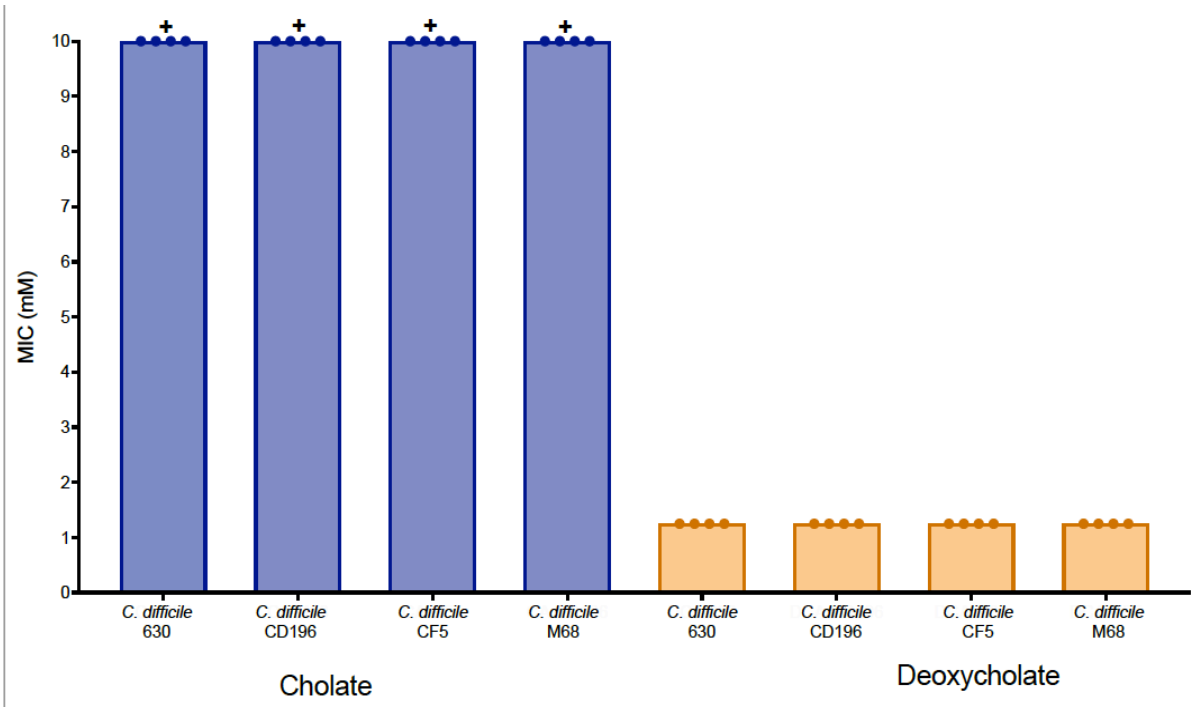
	Cholate (CA)		Deoxycholate (DCA)	
Organism	Average	SD	Average	SD
C. scindens VPI 12708	2.50	0.00	1.88	0.72
C. scindens ATCC 35704	2.50	0.00	2.19	0.63
C. hiranonis	2.50	0.00	0.78	0.31
C. hylemonae	7.00	0.00	1.25	0.00
C. difficile R20291	10.00	0.00	1.56	0.63



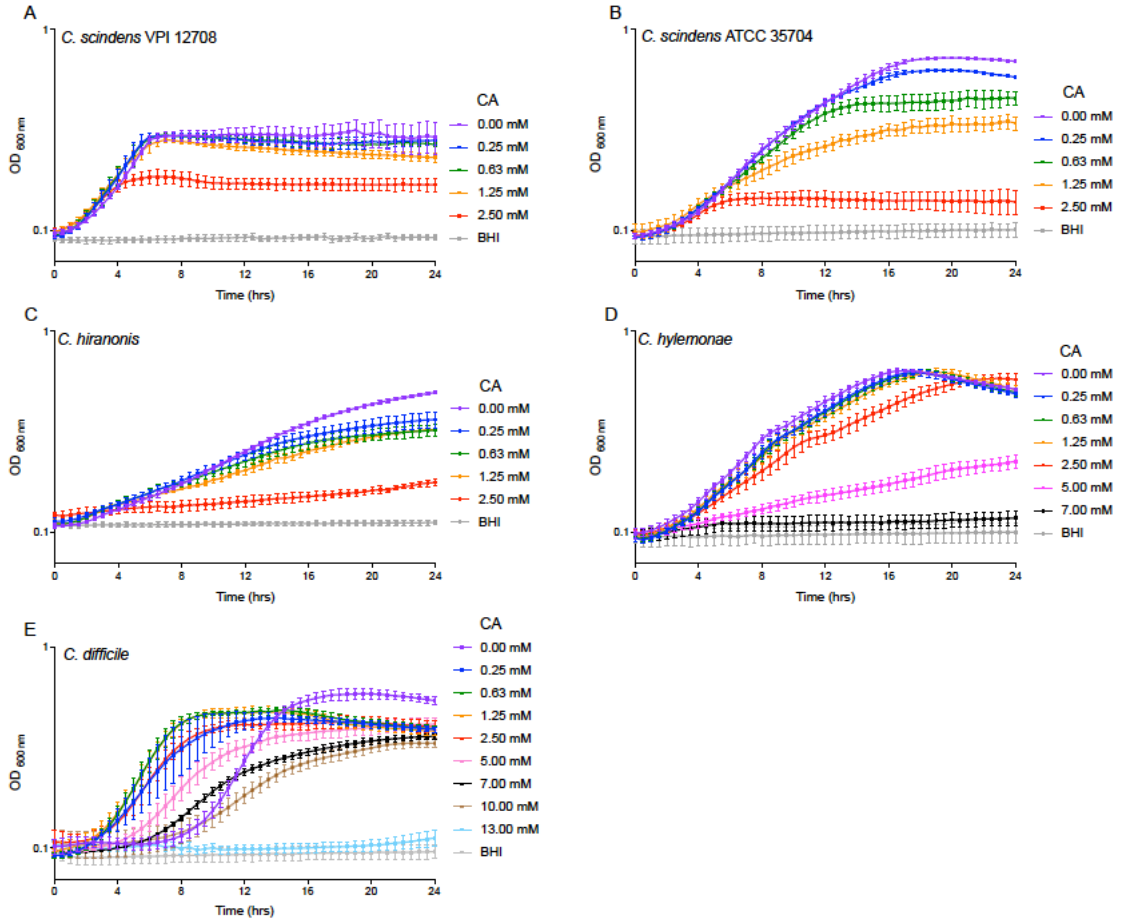
Supplemental Figure 1: Optical density/Colony Forming Units conversion for commensal *Clostridia* and *C. difficile.* Linear regression of OD_{600} plotted against CFU/mL for *C. scindens* **(A)**, *C. hiranonis* **(B)**, *C. hylemonae* **(C)** and *C. difficile* **(D).**



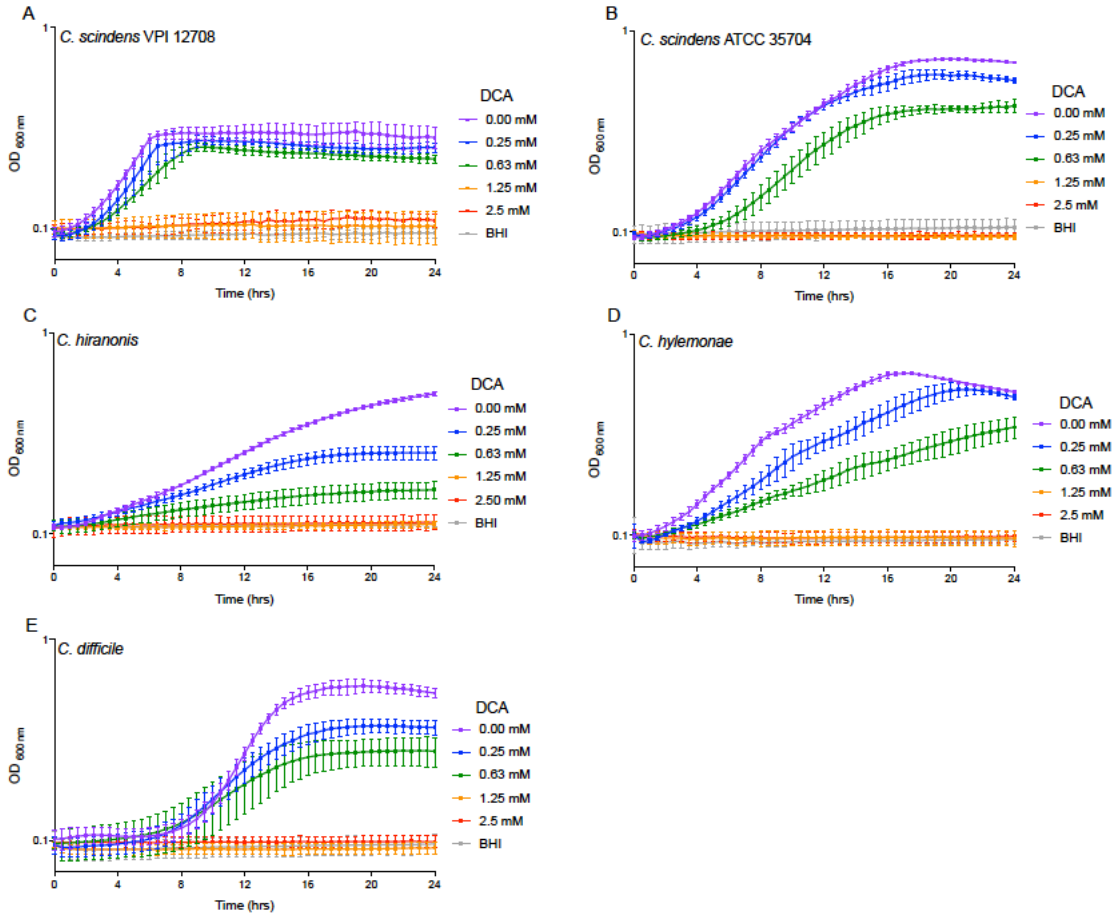
Supplemental Figure 2: Genome comparison of commensal *Clostridia* strains. Whole-genome nucleotide BLAST alignment of *C. scindens* VPI 12708, *C. hiranonis,* and *C. hylemonae* against reference strain *C. scindens* ATCC 35704.



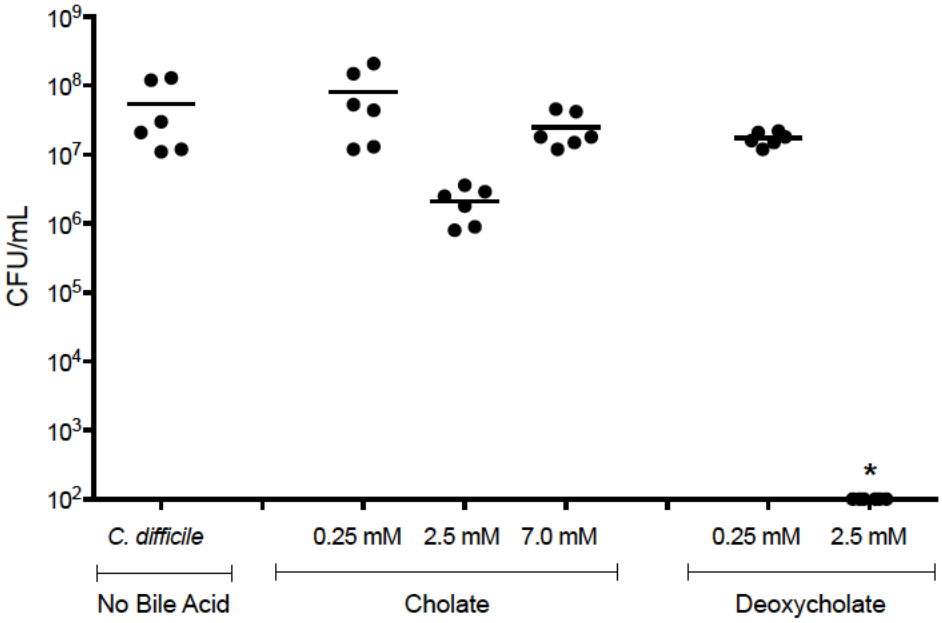
Supplemental Figure 3: MICs for CA and DCA are consistent across different *C. difficile* strains. The Minimum Inhibitory Concentration (MIC) of CA and DCA was tested on *C. difficile* 630, *C. difficile* CD196, *C. difficile* CF5, and *C. difficile* M68. The MIC was defined as the lowest concentration of compound that showed no visible growth. A plus sign (+) was used to note when growth was observed at the highest concentration of bile acid tested. Growth was defined at 24 hr. Four biological replicates were performed.



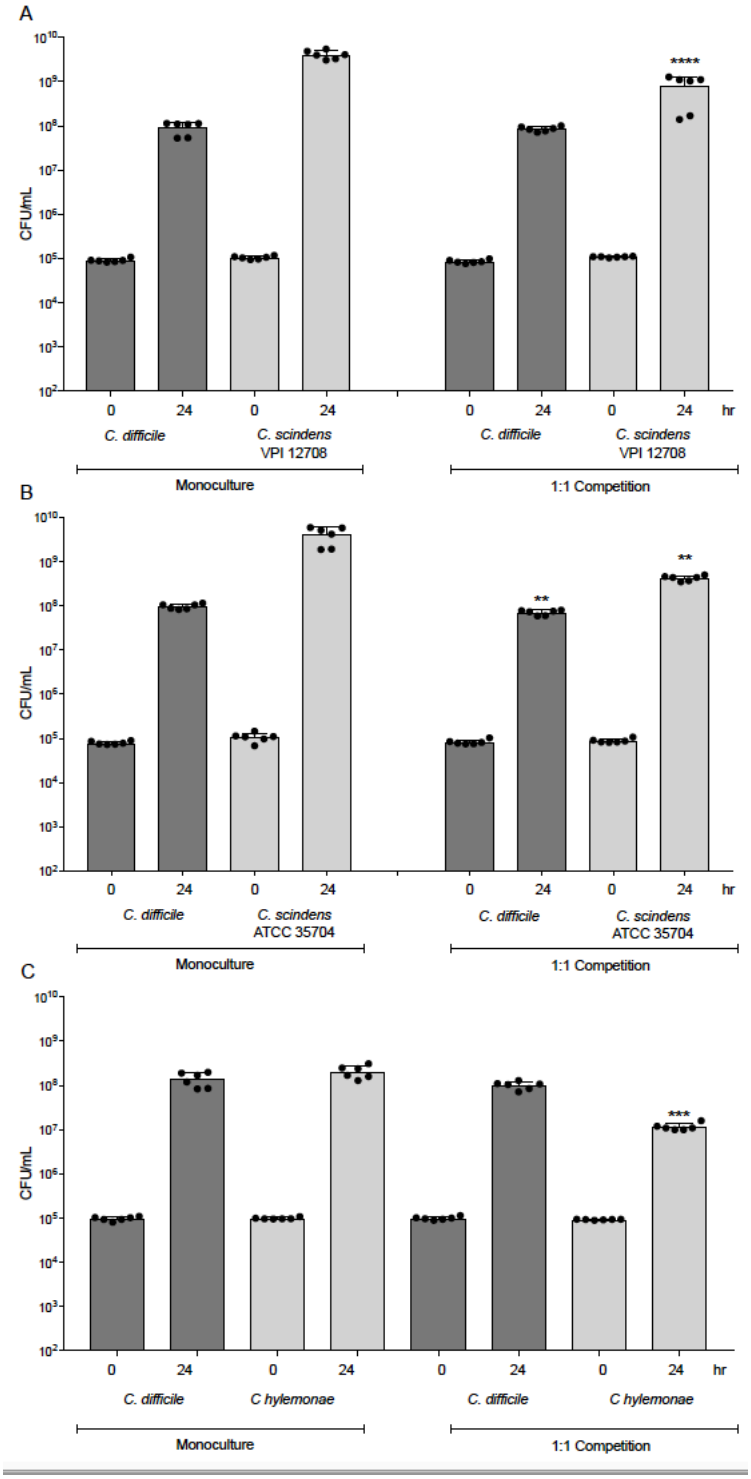
Supplemental Figure 4: Cholate inhibits growth in *Clostridia* strains in a dose dependent manner. Growth of *C. scindens* VPI 12708 **(A)**, *C. scindens* ATCC 35704 **(B),** *C. hiranonis* **(C)**, *C. hylemonae* **(D)**, and *C. difficile* **(E)** in the presence of multiple concentrations of CA along with a no bile acid control over 24 hr.



Supplemental Figure 5: Deoxycholate inhibits growth in *Clostridia* strains in a dose dependent manner. Growth of *C. scindens* VPI 12708 **(A)**, *C. scindens* ATCC 35704 **(B),** *C. hiranonis* **(C)**, *C. hylemonae* **(D)**, and *C. difficile* **(E)** in the presence of multiple concentrations of DCA along with a no bile acid control over 24 hr.



Supplemental Figure 6: Deoxycholate inhibits *C. difficile* at a 2.5 mM concentration. Inhibition of *C. difficile* after 24 hr of growth in media supplemented with 0.25 mM CA, 2.5 mM CA, 7.0 mM CA, 0.25 mM DCA or 2.5 mM of DCA. Experiments were run in duplicate and three biological replicates were performed. Inhibition was measured by comparison to a *C. difficile* control grown in media without bile acid supplementation. Statistical significance was determined by one-way ANOVA with Tukey used for multiple comparisons (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$).



Supplemental Figure 7: *C. difficile* inhibits all commensal *Clostridia* strains except *C. scindens* ATCC 35704 in co-culture. CFU/ml at 0 and 24 hr time points for monoculture and 1:1 competition with *C. difficile* for *C. scindens* VPI 12708 **(A)**, *C. scindens* ATCC 35704 **(B)**, and *C. hylemonae* **(C)**. Statistical significance was determined by one-way ANOVA with Tukey used for multiple comparisons (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$).

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Chapter 3: The differential metabolic response to hydroxyproline by *C. difficile* and commensal *Clostridia*

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Abstract

An intact gut microbiota confers colonization resistance against *Clostridioides difficile* through a variety of mechanisms, likely including competition for nutrients. Recently, proline was identified as an important environmental amino acid that *C. difficile* uses to support growth and cause significant disease. A post-translationally modified form, trans-4-hydroxyproline, is highly abundant in collagen, which is degraded by host proteases in response to *C. difficile* toxin activity. The ability to dehydrate trans-4-hydroxyproline via the HypD glycyl radical enzyme is widespread amongst gut microbiota, including *C. difficile* and members of the commensal *Clostridia*, suggesting that this amino acid is an important nutrient in the host environment. Therefore, we constructed a *C. difficile* Δ*hypD* mutant and found that it was modestly impaired in fitness in a mouse model of infection, and was associated with an altered microbiota when compared to mice challenged with the wild type strain. Changes in the microbiota between the two groups were largely driven by members of the *Lachnospiraceae* family and the *Clostridium* genus. We found that *C. difficile* and type strains of three commensal Clostridia had significant alterations to their metabolic gene expression in the presence of trans-4-hydroxyproline *in vitro*. The proline

reductase (*prd*) genes were elevated in *C. difficile*, consistent with the hypothesis that trans-4-hydroxyproline is used by *C. difficile* to supply proline for fermentation. Similar transcripts were also elevated in some commensal *Clostridia* tested, although each strain responded differently. This suggests that the uptake and utilization of other nutrients by the commensal *Clostridia* may be affected by trans-4-hydroxyproline metabolism, highlighting how a common nutrient may be a signal to each organism to adapt to a unique niche. Further elucidation of the differences between them in the presence of hydroxyproline and other key nutrients will be important to determining their role in nutrient competition against *C. difficile.*

Introduction

Clostridioides difficile infection (CDI) is the cause of significant morbidity and mortality and is responsible for over 4.8 billion dollars in excess medical costs each year[1, 2]. The current front-line treatment for CDI is the antibiotic vancomycin, which can resolve CDI[3]. However, 20-30% of patients will experience a recurrence of CDI within 30 days, and 40-60% of the patients who have experienced one recurrence will have multiple recurrences[4, 5]. The use of antibiotics, including vancomycin, is a major risk factor for CDI due to their effect on the gut microbiota, which causes a loss of colonization resistance against *C. difficile*[6-8]. Colonization resistance, or the ability of the gut microbiota to defend against colonization by gastrointestinal pathogens such as *C. difficile* has many potential mechanisms, including the production of inhibitory metabolites and competition for nutrient sources[9-11]. Conversely, *C. difficile* toxin activity is associated with altered recovery of the gut microbiota, as well as liberation of numerous sugars and peptides/amino acids *in vivo*[12-15]. However, it is unknown if *C. difficile* has a hierarchy of preferred nutrient sources in a host, or whether members of the microbiota also utilize similar nutrients, and if they do, whether their use contributes to colonization resistance.

Much of the research on colonization resistance against *C. difficile* has focused on the effects of secondary bile acids produced by the gut microbiota[16-20]. While secondary bile acid metabolism is an important contributor, other factors such as competition for nutrients are also likely to play a role. For example, colonization of a host by non-toxigenic *C. difficile* can prevent colonization by toxigenic *C. difficile,* indicating that bacteria with similar nutritional needs can occupy an exclusive niche[21, 22]. In addition, the increased amount of succinate available in the antibiotic treated gut promotes expansion by *C. difficile,* indicating that the depletion of the microbiota that occurs after antibiotic use creates a beneficial environment for *C. difficile* colonization and expansion[23]. Metabolic and transcriptomic analysis have also shown that the availability of amino acids and other nutrients is very important in the early stage of CDI[15, 23, 24]. Additionally, the degradation of collagen by host proteases that is induced by *C. difficile* toxin activity may be a source of peptides and amino acids to support *C. difficile* growth throughout the course of infection[14, 15].

C. difficile uses proline as an electron acceptor for Stickland fermentation for energy production and regeneration of NAD^+, yet it does not grow well without the presence of proline and other amino acids important to Stickland fermentation, therefore it must compete for them within the host environment[25-27]. The concentration of proline in media affects expression of genes in the *prd* operon, which encodes proline reductase and accessory proteins, with maximal expression observed when proline content is high[26]. In addition, the availability of proline and branched chain amino acids in the gut correlates with increased susceptibility to *C. difficile* in a mouse model of infection[13]. When a *C. difficile prdB* mutant that was unable to utilize proline as an energy source was tested in a mouse model of CDI, it was less fit *in vivo* and resulted in less toxin (TcdB) in stools when compared to mice challenged with wild type *C difficile*[13]. In addition,

the presence of some commensal *Clostridia* causes an increase in the reliance of *C. difficile* on proline fermentation [28]. This indicates that *C. difficile* may compete with some commensal *Clostridia* for proline in the gut. *C. difficile* also has a competitive advantage over the commensals *Clostridium scindens, Clostridium hylemonae,* and *Clostridium hiranonis* in a rich medium, although the extent to which this is due to the ability of *C. difficile* to ferment proline is unknown[16].

Trans-4-hydroxy-L-proline (hydroxyproline or hyp) is a derivative of proline that has been post-translationally modified by the host via prolyl-4-hydroxylase, and is a significant component of the highly abundant host protein collagen. Recently, we have shown that inflammation resulting from *C. difficile* toxin activity leads to increased expression of host matrix metalloproteinases and subsequent degradation of collagen, likely supplying *C. difficile* with hydroxyproline and other Stickland substrates[14]. *C. difficile* can reduce Hyp to proline in a two-step process that requires the glycyl radical enzyme 4-hydroxyproline dehydratase (HypD) and a pyrroline-5-carboxylate reductase (P5CR) encoded by the gene *proC* [28-30]. Homologs of HypD are widespread in the gut microbiome, and a subset of organisms, largely *Clostridia*, that carry the *hypD* gene also encode an adjacent P5CR homolog, indicating that the ability of bacteria to reduce hydroxyproline may be useful in the gut[29]. The widespread presence of HypD and the competitive fitness advantage gained by proline fermentation indicates that the ability to ferment proline may play a significant role in *C. difficile* colonization in the gut[29, 31].

In this study, we hypothesized that use of hydroxyproline by *C. difficile* contributes to its fitness *in vivo*. We tested this by examining disease kinetics of wild type (WT) *C. difficile* and a Δ*hypD* mutant in a mouse model of CDI. Mice challenged with the Δ*hypD* mutant had reduced weight loss, less toxin activity, and increased relative abundances of cecal *Lachnospiraceae,* a

family which includes many commensal *Clostridia,* as well as members of the *Clostridium* genus. We also show that hydroxyproline affects the transcriptomes of *C. difficile* and three commensal *Clostridia* species (*C. scindens*, *C. hylemonae*, and *C. hiranonis*), though each had unique gene expression profiles, with alterations to pathways for carbohydrate and amino acid utilization among them. Together, these data show that *C. difficile* relies on hydroxyproline metabolism *in vivo* for robust sporulation and toxin production. Further, it identifies numerous metabolic pathways in *C. difficile* and commensal Clostridia that are affected by hydroxyproline, and the unique response of each organism indicates that hydroxyproline may act as a nutrient source and a signal to prime them for metabolism of other specific nutrients.

Methods

Animals and housing. C57BL/6J WT mice (5–8 weeks old; n = 18 male and n = 18 female) were purchased from Jackson Labs. The food, bedding, and water were autoclaved, and all cage changes were performed in a laminar flow hood. The mice were subjected to a 12 h light and 12 h dark cycle. Mice were housed in a room with a temperature of 70 F and 35% humidity. Animal experiments were conducted in the Laboratory Animal Facilities located on the NCSU CVM campus. Animal studies were approved by NC State's Institutional Animal Care and Use Committee (IACUC). The animal facilities are equipped with a full-time animal care staff coordinated by the Laboratory Animal Resources (LAR) division at NCSU. The NCSU CVM is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC). Trained animal handlers in the facility fed and assessed the status of animals several times per day. Those assessed as moribund were humanely euthanized by CO2 asphyxiation.

Mouse model of *C. difficile* infection. The mice were given 0.5 mg/mL cefoperazone in their

drinking water for 5 days to make them susceptible to *C. difficile* infection, then plain water for 2 days, after which time they (n =8, 4 males and 4 females) received 10^5 spores of either *C. difficile* 630Δ*erm* (WT) or *C. difficile* 630Δ*erm*Δ*hypD* (*ΔhypD*) via oral gavage. One group of mice (n = 8, 4 males and 4 females) received antibiotics and no *C. difficile* spores (cef) and were used as uninfected controls. Mice were weighed daily and monitored for clinical signs of distress (ruffled fur, hunched posture, slow ambulation, etc.). Fecal pellets were collected 1-, 3-, 5-, and 7-days post challenge and diluted 1:10 w/v in sterile PBS, then serially diluted in 96-well PCR plates and plated onto CCFA for enumeration of vegetative *C. difficile* CFU. The serially diluted samples were then removed from the anaerobic chamber and heated to 65°C for 20 min before being passed back into the chamber. The dilutions were plated onto TCCFA for enumeration of spore CFUs. Additional fecal pellets were collected on days 1-7 and stored at -80°C for later use in toxin activity assays and 16S rRNA sequencing.

At day 7 post challenge, mice were humanely sacrificed, and necropsy was performed. Cecal content was harvested for enumeration of vegetative *C. difficile* and spore CFUs, as well as for toxin activity. Cecal tissue was harvested for 16S rRNA sequencing. Samples for sequencing and toxin activity were immediately flash frozen in liquid nitrogen and stored at −80°C until processing.

Toxin activity in the cecal content was quantified using the Vero Cell cytotoxicity assay[32]. Briefly, the content was diluted 1:10 w/v in sterile PBS, and 10-fold dilutions were added to Vero cells in a 96-well dish for ~16 h. The reciprocal of the lowest dilution in which ~80% of the cells have rounded was reported as the titer.

Construction of *C. difficile* strains. To construct the pMTL-YN1C-*hypD* complementation construct, primer pair YH-P295 and YH-P296 was used to amplify the *hypD* gene (Supplemental

Table 1). The resulting PCR product was digested with NotI and XhoI and ligated to pMTL-YN1C digested with the same enzymes. The resulting PCR fragments were inserted into pMTL-YN1C digested with NotI and XhoI using Gibson assembly [33]. The assembly mixture was transformed into *E. coli* DH5α, and the resulting plasmids were confirmed by sequencing and then transformed into *E. coli* HB101/pRK24.

Vectors for gene deletion and complemention. To construct the pMTL-YN3-Δ*hypD* allelic exchange construct, vector~1 kb flanking regions of *hypD* (CD630_32820) were PCR amplified using primers YH-P253 and YH-P25 were used to amplify the region 4(upstream of *hypD*,) and primers YH-254 and YH-256 were used to amplify a region downstream of *hypD* using *C. difficile* 630 genomic DNA as the template. The resulting PCR products were used in a PCR splice overlap extension (SOE) reaction with the flanking primers YH-257 and YH259. To construct the pMTL-YN3-Δ*p5cr* allelic exchange construct, primers YH-P258 and YH-P260 were used to amplify a region upstream of *p5cr*, and primers YH-254 and YH-256 were used to amplify a region downstream of *p5cr* in *C. difficile* (Supplemental Table 1). To construct the pMTL-YN3-Δ *p5cr* allelic exchange vector, ~1 kb flanking regions of *proC* (CD630_32810) were PCR amplified using primers YH-P257 and YH-P258 (upstream) and primers YH-259 and YH-260 (downstream) with *C. difficile* 630 genomic DNA as the template (Supplemental Table 1). All PCRs of flanking regions were carried out using Phusion-HF Master Mix (NEB) according to the manufacturer's protocol with an annealing temperature (Ta) of 61°C and extension time of 25 sec. gel-purified using illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare). 30 ng of each upstream and downstream flanking region of the targeted gene were used as templates for overlap. PCR products were used in a10 µl reactions (Ta of 72°C, extension time of 120s). 7 µl of each overlap PCR mix was used as template in 20 µl extension PCRs (Ta of 61°C, extension time of 60

sec, Phusion-HF master mix) with 0.75 µM of each flanking primer YH-P253 and P256 for Δ*hypD,* and YH-P257 and YH-P260 for Δ*proC* to amplify joined flanking regions. The PCR SOE products were gel-purified and digested with AscI and SbfI. The assembly-HF (NEB). was linearized-HF and gel-purified. Each deletion region was ligated into linearized pMTL-YN3 using T4 DNA ligase (NEB) in 10 µl reactions at a 1:9 volume ratio (vector:insert).Ligation reactions were *E. coli* TOP10 cells and plated out onto LB-chloramphenicol (25 µg/mL) agar plates. To construct the pMTL-YN1C-*hypD* complementation construct, the promoter region, ~300bp, upstream of the HypD activase gene (CD630_32830) and *hypD* were separately amplified using primers YH-P229-P230 (Ta of 63°C, extension time of 10 sec) and YH-P231-P232 (Ta of 59°C, extension time of 60 sec), respectively (Supplemental Table 1). To construct the pMTL-YN1C-*proC* complementation construct, primers YH-P233 and YH-P234 was used to amplify the P5CR-encoding gene along with ~200 bp of the upstream region using a Ta of 60°C, extension time of 20 sec (Supplemental Table 1). The resulting PCR products were gel-purified. 3-fold molar excess of each PCR insert was Gibson assembled into 50 ng or 100 ng of StuI-linearized pMTL-YN1C to construct pMTL-YN1C-*hypD* and pMTL-YN1C-*proC*, respectively. Gibson assembly reactions were transformed into *E. coli* DH5α,TOP10 cells and then plated out onto LB-chloramphenicol (25 µg/mL) agar plates. The resulting plasmids were confirmed by Sanger sequencing and then transformed into *E. coli* HB101/pRK24 for conjugation.

Gene deletions in *C. difficile*. Allele-coupled exchange was used to construct clean deletions of *hypD* and *PC5R* [34]. The recipient *C. difficile* strain 630Δ*erm*Δ*pyrE* (a kind gift from Nigel Minton, c/o Marcin Dembek) was grown for 5-6 hrs in BHIS medium in an anaerobic chamber (Coy, USA) *E. coli* HB101/pRK24 donor strains carrying the appropriate pMTL-YN3 allelic exchange constructs were grown in LB medium containing ampicillin (50 µg/mL) and

chloramphenicol (20 μg/mL) at 37˚C°, 225 rpm, under aerobic conditions, for 5-6 hrs. Each *E. coli* strain was pelleted at 2,500 rpm for 5 min and transferred into an anaerobic chamber. One milliliter of the *C. difficile* culture was added to each *E. coli* pellet, and 100 μL of the mixture was spotted seven times onto a BHIS plate. The *E. coli* and *C. difficile* mixture was incubated for 13-18 hrs at 37˚C anaerobically after which the resulting growth was scraped from the plate into 1 mL phosphate buffered saline (PBS). One hundred microliter aliquots of each suspension were spread onto five BHIS plates containing 10 μg/mL thiamphenicol, 50 μg/mL kanamycin, and 8 μg/mL cefoxitin. The plates were incubated for 3-4 days at 37˚C, and transconjugants were passaged onto BHIS plates containing 15 μg/mL thiamphenicol, 50 μg/mL kanamycin, 8 μg/mL cefoxitin, and 5 μg/mL uracil. After selecting for the fastest growing colonies over 2-3 passages, single colonies were re-struck onto CDMM plates, a defined minimal medium, containing 2 mg/mL 5-fluoroorotic acid (FOA) and 5 μg/mL uracil. FOA-resistant colonies that arose were patched onto CDMM plates containing 5-FOA and uracil, and colony PCR was performed to identify clones harboring the desired deletions[35]. (Supplementary Table 1) All 630Δ*erm*Δ*pyrE* mutant strains were complemented with *pyrE* in the *pyrE* locus as described in the next section.

Complementation in *C. difficile*. *E. coli* HB101/pRK24 donor strains carrying the appropriate complementation construct were grown in LB containing ampicillin (50 μg/mL) and chloramphenicol (20 μg/mL) at 37˚C°, 225 rpm, under aerobic conditions, for 6 hrs. [34, 35].For complementation in the *pyrE* locus using pMTL-YN1C constructs,*C. difficile* recipient strains were conjugated with either the empty pMTL-YN1C vector or the appropriate pMTL-YN1C complementation vectors as described previously . Transconjugants were then re-struck onto CDMM and incubated for 2-4 days. Colonies that had restored the *pyrE* locus by virtue of their ability to grow on CDMM were re-struck onto CDMM plates before further characterization. All

clones were verified by colony PCR. At least two independent clones from each complementation strain were phenotypically characterized.

Bacterial strain collection and growth conditions. The *C. difficile* strains used in this study were the wild type *C. difficile* 630Δ*erm* (WT) and the mutants *C. difficile* 630Δ*erm*Δ*hypD* (Δ*hypD*), *C. difficile* 630Δ*erm*Δ*p5cR* (Δ*p5cR*), *C. difficile* 630Δ*erm*Δ*hypD::hypD* (*hypD* complement), and *C. difficile* 630Δ*erm*Δ*p5cR::p5cR* (*p5cR* complement). All assays using *C. difficile* were started from spore stocks, which were prepared and tested for purity as described previously [36, 37]. *C. difficile* spores were maintained on brain heart infusion (BHI) media supplemented with 100 mg/L L-cysteine and 0.1% taurocholate (T4009, Sigma-Aldrich). Then cultures were started by inoculating a single colony from the plate into BHI liquid media supplemented with 100 mg/L L-cysteine. The other bacterial strains used in this study were *C. hiranonis* TO 931*, C. hylemonae* TN 271, and *C. scindens* VPI 12708. All strains were maintained on 15% glycerol stocks stored in –80°C until use and were grown in BHI medium supplemented with 100 mg/L L-cysteine. All strains used in this study were grown under 2.5% hydrogen under anaerobic conditions (Coy, USA) at 37°C.

Growth studies in CDMM. *C. difficile* was grown in a well-established, defined minimal media (CDMM)[26]. CDMM –pro +hyp had 600 mg/L of trans-4-hydroxyproline (Sigma) instead of proline. CDMM –pro was used as a negative control. A single colony was inoculated into 5 mL of culture and incubated at 37°C for 24 hr, at which point the OD_{600} was measured using a spectrophotometer.

Genomic analysis of *hypD* and *p5cR*. This was performed using the gggenes R package and Geneious as described previously[16]. Briefly, the *hypD* comparison was constructed by first extracting the positional information for *hypD* and *p5cR* from Geneious [38], then obtaining amino acid identity percentage through BLASTp alignments [39] against coding sequences from the

reference strain *C. scindens* ATCC 35704. This data was visualized using the publicly available gggenes R package [40].

RNA extraction. *C. difficile, C. scindens, C. hiranonis* and *C. hylemonae* liquid cultures were started from a single colony and grown in either BHI or BHI + 600 mg/L hydroxyproline for 14 hr before RNA extraction. Cultures were fixed by adding equal volumes of a 1:1 mixture of EtOH and acetone and stored at -80ºC for later RNA extraction. For extraction, the culture was thawed, then centrifuged at 10,000 rpm for 10 min at 4ºC. The supernatant was discarded and the cell pellet resuspended in 1 mL of 1:100 BME:H_2O, then spun down at 14,000 rpm for 1 min. For RNA to be used for qRT-PCR, the cell pellet was resuspended in 0.3 mL of lysis buffer from the Ambion RNA purification kit (AM1912, Invitrogen) then sonicated while on ice for 10 pulses of 2 sec with a pause of 3 sec between each pulse. Extraction was then performed following the manufacturers protocol from the Ambion RNA purification kit. For RNA to be used for RNA-seq, the cell pellet was resuspended in 1 mL of Trizol (Thermofisher) and incubated at room temperature for 15 min. 200 µL of chloroform (Sigma-Aldrich) was added, the solution was inverted rapidly for 20 sec, then incubated at room temperature for 15 min and centrifuged at 14,000 rpm for 15 min at 4ºC. The aqueous phase was mixed with 96% ethanol and the extraction was performed using the Direct-zol RNA Miniprep Plus following the manufacturer's instructions, including an on-column DNase I treatment (R2071, Zymo Research).

Reverse transcription and quantitative real-time PCR. Reverse transcription and quantitative real-time PCR was performed as described previously [15]. Briefly, RNA was depleted by using Turbo DNase according to the manufacturer's instructions (AM2238, Invitrogen. The DNase-treated RNA was then cleaned using an RNA clean up kit (R1019, Zymo) according to manufacturer's instructions and DNA depletion was verified by amplifying 1 µL of RNA in a PCR

reaction. The DNA depleted RNA was used as the template for reverse transcription performed with Moloney murine leukemia virus (MMLV) reverse transcriptase (M0253, NEB). The cDNA samples were then diluted 1:4 in water and used in quantitative real-time PCR with gene-specific primers using SsoAdvanced Universal Sybr green Supermix (1725271, Bio-Rad) according to the manufacturer's protocol. Amplifications were performed in technical triplicate, and copy numbers were calculated using a standard curve and normalized to that of a housekeeping gene. *gyrA* was the housekeeping gene used for *C. scindens*, while *rpoC* was used for *C. difficile, C. hiranonis* and *C. hylemonae.* Primers used can be found in Supplemental Table 2

RNAseq. Sequencing of RNA derived from *in vitro* cultures was performed at the Roy J. Carver Biotechnology Center at the University of Illinois at Urbana-Champaign. Ribosomal RNA was removed from the samples using the RiboZero Epidemiology Kit. (Illumina). RNAseq libraries were prepped with the TruSeq Stranded mRNA Sample Prep Kit (Illumina), though poly-A enrichment was omitted. Library quantification was done via qPCR, and the samples were sequenced on one lane for 151 cycles from each end of the fragments on a NovaSeq 6000 using a NovaSeq S4 reagent kit. The FASTQ files were generated and demultiplexed using the bcl2fastq v2.20 Conversion Software (Illumina). Raw paired Illumina reads were imported into Geneious 10.2.6, where adapters were removed using BBDuk with a Kmer length of 27. The reads were mapped to the *C. difficile* 630*Δerm* genome (NCBI accession no. NC_009089.1), the *C. hiranonis* DSM 13275 genome (NCBI accession no. GCA_008151785.1), the *C. hylemonae* DSM 15053 genome (NCBI accession no. PRJNA523213), or the *C. scindens* ATCC 35704 genome (NCBI accession no. PRJNA508260) using BBMap with a Kmer length of 10 and no other changes to the default settings. Differential analysis was performed using DESeq2 and if genes had an adjusted p value of <0.05 and ± 1 log fold change, they were considered differentially expressed. Kegg

pathway analysis of differentially expressed genes was performed using KEGGREST[41], and Kegg pathway analysis barplots were generated in Graphpad Prism 8. Visualization of differentially enriched genes for each organism was performed using pheatmap (version 1.0.12), ggplots (version 3.3.4), and ggpubr (version 0.4.0.999) within R (version 3.6.3). Some of the differentially enriched genes were hypothetical proteins, those results were removed before the figures were visualized. Full RNAseq data is available in Supplemental File 1.

Metabolomics data analysis.

Mass spectrometry data acquisition. Samples were diluted 1:100 (10 µL sample, 990 µL water) and transferred to an autosampler vial for analysis by UPLC-MS. For quantification of amino acids, a certified reference material amino acid mix solution (*Trace*CERT, Sigma) was diluted to achieve a 100 µM working standard solution. Ten calibration standards ranging from 100 µM to 250 nM were prepared by serially diluting the working standard solution. For quantification of hydroxyproline and 5-aminovaleric acid, certified reference material (Sigma) for each was suspended in water to achieve a 1 mg/mL solution which were combined and diluted to achieve a 50 µg/mL working standard solution. Ten calibration standards ranging from 50 µg/mL to 25 ng/mL were prepared by serially diluting the working standard solution. The analysis was performed using a Thermo Vanquish UPLC instrument (Thermo Fisher Scientific, Germering, Germany) coupled to a Thermo Orbitrap Exploris 480 mass spectrometer (Thermo Fisher Scientific, Breman, Germany) with a heated electrospray ionization (HESI) source. Chromatographic separation was achieved on a Waters BEH Amide column (2.1 x 100 mm, 1.8 µM) maintained at 45°C. The following linear gradient of mobile phase A (H_2O + 0.1% FA) and mobile phase B (MeCN + 0.1% FA) was used: 0-0.1 min (99%B, 0.4 mL/min), 0.1-7 min (99-30%B, 0.4 mL/min), 7-10 min (99%B, 0.4 mL/min). Samples were analyzed (2 µL injections) in

positive ion mode (spray voltage 3.5 kV, ion transfer tube temperature 300°C, vaporizer temperature 350ºC, sheath gas 50 a.u., aux gas 10 a.u., sweep gas 1 a.u.) with a mass range of m/z 60-1000. MS1 data was collected with a resolving power of 60,000 and an AGC target of 1e6 and ddMS2 data was collected with a resolving power of 30,000, cycle time of 0.6 s, AGC target of 4e5 and stepped HCD collision energy (30, 50, 150). The full data set was acquired in a randomized fashion with water blanks and system suitability samples (QReSS, Cambridge Isotope Laboratories) collected every 10 samples.

Targeted data processing. Peak integration and amino acid quantification were performed in Skyline[1]. Individual standard curves for each of the 16 amino acids plus hydroxyproline and 5-aminovalerate were constructed using extracted ion chromatogram peak areas from MS1 data and the slope of each curve was calculated using a linear curve fit and a 1/(x *x) weighting. MS2 data was utilized to validate amino acid annotations, particularly to differentiate valine and 5-aminovaleric acid. The concentrations in the study samples were calculated in an identical manner relative to the regression line. Calibration curves for each of the amino acids had R^2 values ranging from 0.9919 to 0.9994 for the linear range 0.25 to 100 ɥ M. Calibration curves for hydroxyproline and 5-aminovaleric acid had R^2 values of 0.9948 to 0.9997, respectively, for the linear range .025 to 50 ɥ g/mL[42].

16S rRNA bacterial sequencing. Fecal and cecal samples were sequenced by the University of Michigan Microbial Systems Molecular Biology Laboratory using the Illumina MiSeq platform. Microbial DNA was extracted from the fecal and cecal samples using Mag Attract Power Microbiome kit (Mo Bio Laboratories, Inc.). A dual-indexing sequencing strategy was used to amplify the V4 region of the 16S rRNA gene [65]. Each 20-µl PCR mixture contained 2 µl of 10X Accuprime PCR buffer II (Life Technologies, CA, 1 USA), 0.15 µl of Accuprime high-fidelity

polymerase (Life Technologies, CA, USA), 5 µl of a 4.0 µM primer set, 3 µl DNA, and 11.85 µl sterile nuclease free water. The template DNA concentration was 1 to 10 ng/µl for a high bacterial DNA/host DNA ratio. The PCR conditions were as follows: 2 min at 95°C, followed by 30 cycles of 95°C for 20 sec, 55°C for 15 sec, and 72°C for 5 min, followed by 72°C for 10 min. Libraries were normalized using a Life Technologies SequalPrep normalization plate kit as per manufacturer's instructions for sequential elution. The concentration of the pooled samples was determined using the Kapa Biosystems library quantification kit for Illumina platforms (Kapa Biosystems, MA, USA). Agilent Bioanalyzer high sensitivity DNA analysis kit (Agilent CA, USA) was used to determine the sizes of the amplicons in the library. The final library consisted of equal molar amounts from each of the plates, normalized to the pooled plate at the lowest concentration. Sequencing was done on the Illumina MiSeq platform, using a MiSeq reagent kit V2 (Ilumina, CA, USA) with 500 cycles according to the manufacturer's instructions, with modifications [65]. Sequencing libraries were prepared according to Illumina's protocol for preparing libraries for sequencing on the MiSeq (Ilumina, CA, USA) for 2 or 4 nM libraries. PhiX and genomes were added in 16S amplicon sequencing to add diversity. Sequencing reagents were prepared according to the Schloss SOP (https://www.mothur.org/wiki/MiSeq_SOP#Getting_started), and custom read 1, read 2 and index primers were added to the reagent cartridge. FASTQ files were generated for paired end reads.

Community sequencing microbial analysis: Analysis of the V4 region of the 16S rRNA gene was performed in the statistical programming environment R using the DADA2 package (version 1.14.1) [43]. Forward/reverse pairs were trimmed and filtered, with forward reads truncated at 240 nt and reverse reads truncated at 200 nt. No ambiguous bases were allowed, and each read was required to have less than two expected errors based on their quality score. Error corrected ASVs

were independently inferred for the forward and reverse reads of each sample and then read pairs were merged to obtain amplicon sequence variants (ASVs). Chimeric ASVs were identified and removed. For taxonomic assignment ASVs were compared to the Silva v132 database (https://zenodo.org/record/1172783). The R package phyloseq (version 1.30) was used to further analyze and visualize data[44]. Inverse Simpson was the metric used to calculate alpha diversity and Kruskal Wallis was used to determine statistical significance between treatment groups. Relative abundance was calculated using phyloseq and visualized using Prism 7.0c, and differential-abundance analysis between the hypD and WT treatment groups was performed using the Aldex2 package (version 1.18.0) and visualized using the ggplots2 package (version 3.3.4) [44-46].

Stats: Statistical tests were performed using Prism version 7.0c for Mac OSX (GraphPad Software, La Jolla, CA, USA). Statistical significance was determined using Mann-Whitney for CFUs, spores and toxin activity and Kruskal Wallis with Dunns multiple comparisons for mouse weights during infection. Student T Tests with Welch's Correction to account for multiple comparisons were used to analyze other data. Statistical analysis for the 16S and RNAseq results was performed in the R computing environment. Kruskal Wallis was used for alpha diversity, Permanova Adonis in the vegan package (version 2.5-7) was used to test the difference between groups for the beta diversity analysis, and ALDEx2 was used to calculate the differences between treatments using a centered-log-ratio transform of ASV abundance to create an effect size for each ASV[45, 47].

Results

***C. difficile* requires *hypD* for maximum growth in a defined minimal media supplemented with hydroxyproline.** The reduction of trans-4-hydroxy-L-proline (hereafter hydroxyproline) to L-proline is a two-step process requiring *hypD* and *p5rC* (Figure 1A)[30] In order to test the effect of these genes on the ability to grow in media with hydroxyproline substituted for proline, WT *C.*

difficile, Δ*hypD,* Δ*p5rC* and complemented mutants were grown in defined minimal media (CDMM). To test the ability of each mutant to reduce hydroxyproline, they were grown in CDMM with hydroxyproline substituted for proline (CDMM –pro +hyp), and CDMM without proline (CDMM –pro) as a negative control (Figure 1B). The Δ*hypD* mutant had a significant growth

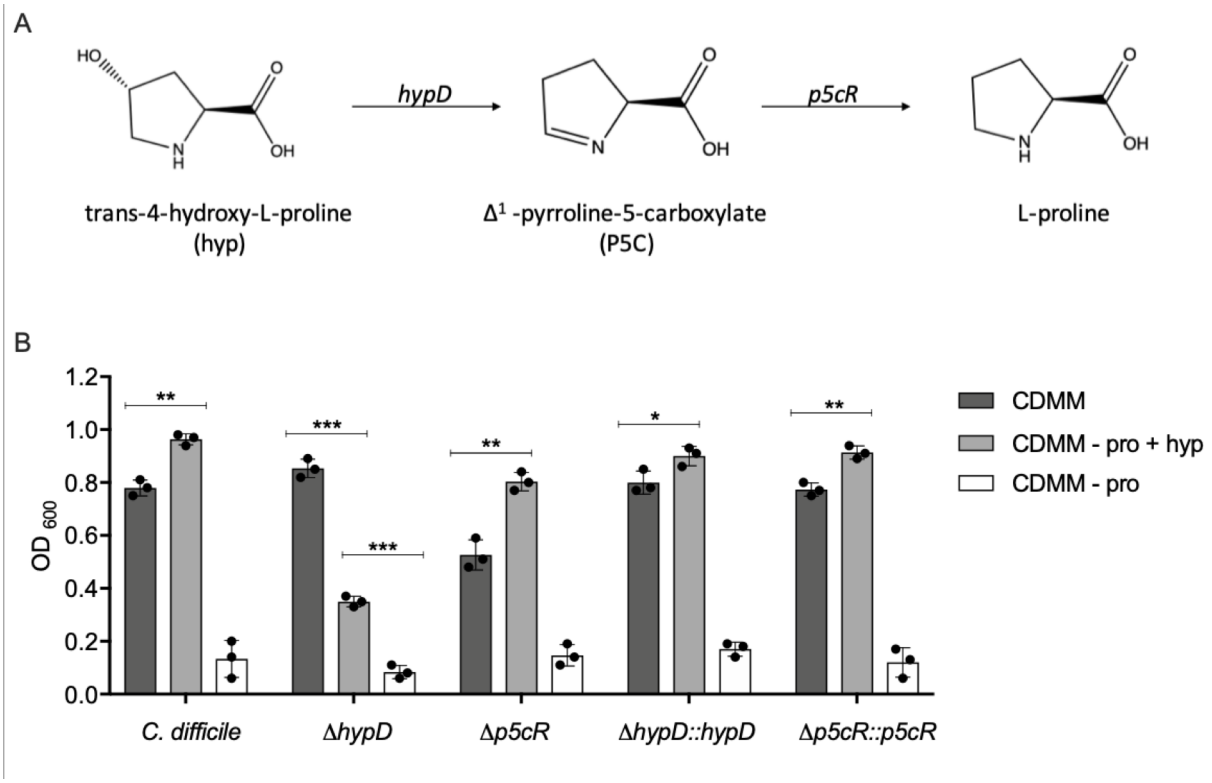

Figure 1: *C. difficile* Δ*hypD* mutant has a growth defect when hydroxyproline is substituted for proline in the growth medium. **A.** Schematic depicting conversion of trans-4-hydroxy-L-proline to L-proline by *hypD* and *p5cR.* **B.** Growth of *C. difficile* 630Δ*erm* WT, the Δ*hypD* and Δ*p5cR* mutants, as well as the complements of both mutants in the defined medium CDMM, in CDMM -proline +hydroxyproline (-pro, +hyp), and CDMM -proline (-pro) Statistical significance was determined using Student's T Test with Welch's Correction to account for multiple comparisons (*, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001).

defect in the CDMM – pro + hyp, indicating that HypD is needed to utilize hydroxyproline (p <0.001, Student's T Test with Welch's correction). There was no growth defect observed in CDMM – pro +hyp for the Δ*p5cR* mutant, indicating that the particular *p5cR* gene tested is not necessary for *C. difficile* to utilize hydroxyproline in the place of proline. Interestingly, the WT

strain as well as Δ*p5cR* and both complements grew significantly better in CDMM – pro +hyp than they did in CDMM alone (<0.05, Student's T Test with Welch's correction). As expected, all strains had very poor growth in CDMM –pro, as proline is essential for *C. difficile* growth.

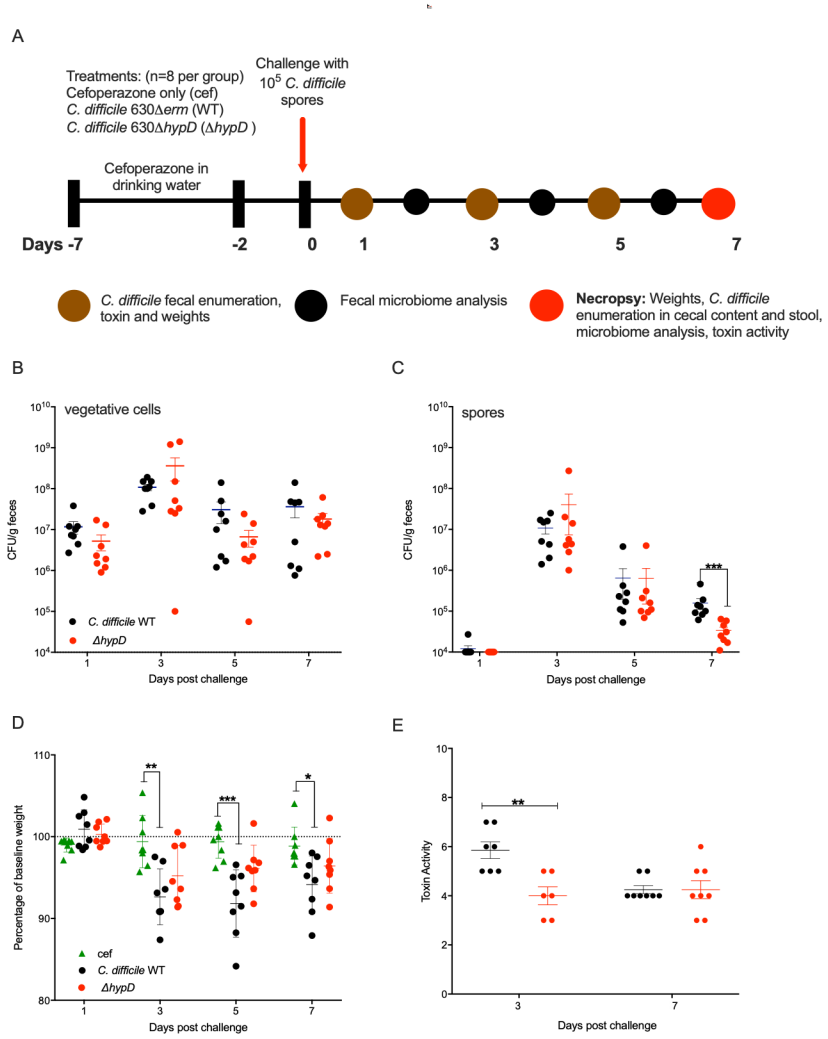

Figure 2: WT *C. difficile* induces more weight loss and toxin activity than a Δ*hypD* mutant in a mouse model of CDI. A. Schematic depicting experimental design. All mice (n=24) received the antibiotic cefoperazone in their drinking water. Subsets of mice were orally challenged with *C. difficile* 630Δ*erm* (WT, n=8) or *C. difficile* 630Δ*hypD* (Δ*hypD,* n=8). The third group of mice were only treated with the antibiotic (cef, n=8). **B-C.** *C. difficile* vegetative cell (**B**) or spore (**C**) CFUs in feces on days 1, 3, 5 and 7 post challenge. **D.** Mouse weights shown as a percentage of baseline (Day 0) weight 1-, 3-, 5- and 7-days post challenge. **E.** Statistical significance was determined using Mann-Whitney (*, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001).

The presence of *hypD* affects weight loss and toxin activity in a mouse model of CDI. To determine how the presence and absence of *hypD* affects the course of CDI, WT C57BL/6J mice (n=8 per group) were challenged with 10^5 spores of WT *C. difficile* or Δ*hypD*, and colonization and disease progression were measured for 7 days (Figure 2A). There was no significant difference in *C. difficile* bacterial load in the feces during infection (Figure 2B), but there was a significant decrease in fecal Δ*hypD* spores when compared to the WT group on day 7 post challenge (Figure 2B, $p < 0.001$, Mann-Whitney). On day 7, bacterial enumeration of cecal content showed no significant difference in the level of *C. difficile* spores (Supplemental Figure 1B), while the *C. difficile* vegetative cells were significantly higher in mice challenged with Δ*hypD* ($p < 0.05$, Mann-Whitney). The biggest difference between the groups was seen in the weights of the mice throughout CDI. WT mice weighed significantly less than the cefoperazone control group (Cef) on days 3 ($p < 0.01$), 5 ($p < 0.001$), and 7 ($p < 0.05$) post challenge (Figure 2D, Kruskal-Wallis with Dunn's multiple comparisons). There was no significant difference in weights between the Δ*hypD* group and the cefoperazone control group, indicating that the WT mice had increased clinical signs of disease compared to the Δ*hypD* group. This finding correlated with high toxin activity from the mice in the WT group compared to the Δ*hypD* group on Day 3 post challenge (Figure 2E, $p<0.01$, Mann-Whitney), although the difference was not significant by Day 7.

Differences in the microbiota between mice challenged with WT *C. difficile* and Δ*hypD* are driven by members of the *Lachnospiraceae* Family. To elucidate the reason behind the observed differences in CDI between mice challenged with WT and Δ*hypD*, the fecal microbiota of the cef, WT, and Δ*hypD* mice was analyzed on day 0 as well as on days 2, 4 and 6 post challenge. The cecal

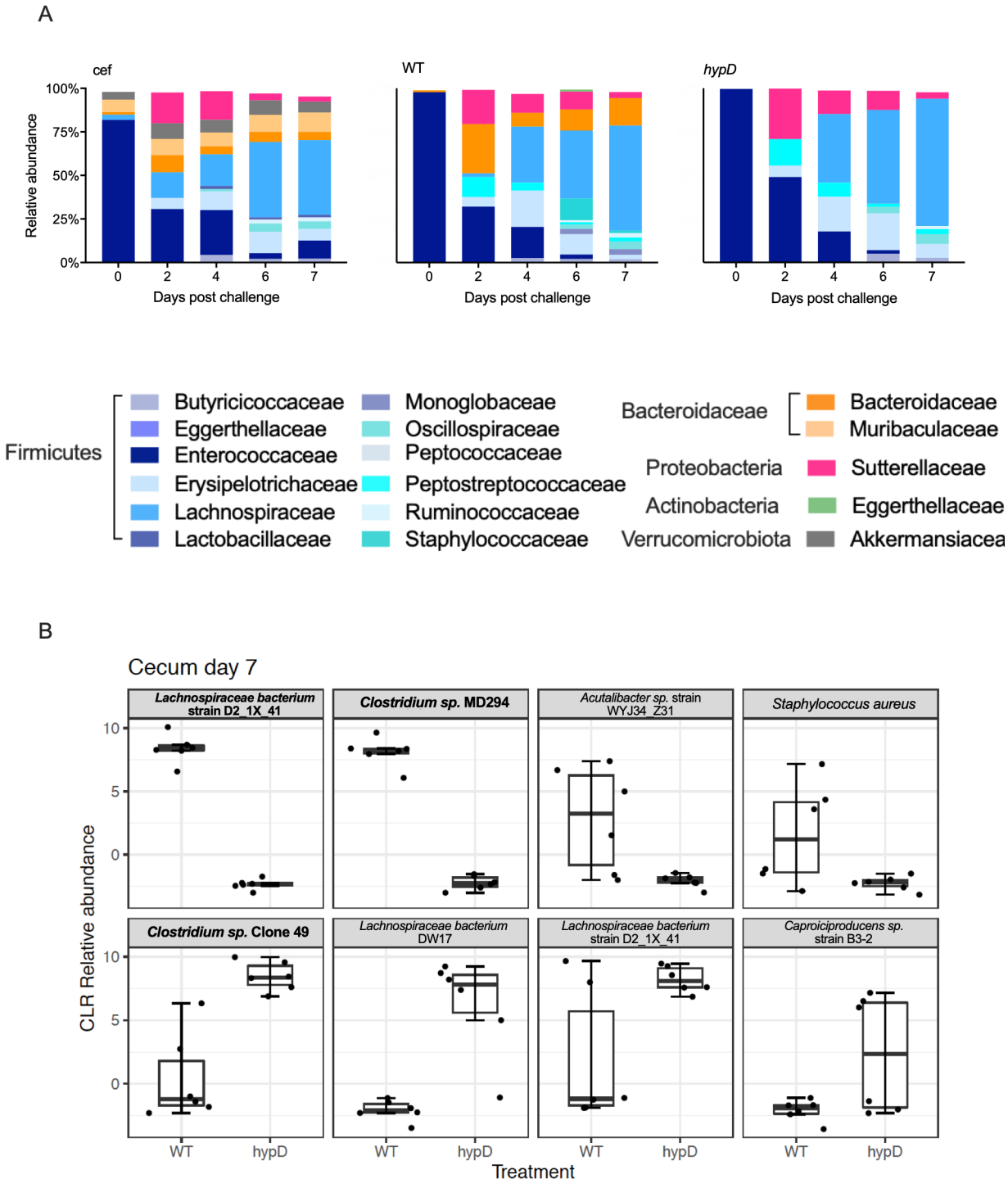

Figure 3: The *Lachnospiraceae* are important when determining the difference between the WT and Δ*hypD* microbiota. A. Relative abundance at the family level for cef, *hypD* and WT fecal microbiome for days 0, 2, 4, and 6 post challenge and the cecal microbiome for day 7 post challenge. **B.** Top 8 ASVs driving differences between *hypD* and WT cecal microbiome on day 7 post challenge. Significant ASVs (q <0.1) are bolded.

microbiota was analyzed on day 7 post challenge, when necropsy occurred. When the alpha diversity was analyzed at the Family level, there were significant differences between the cef and Δ*hypD* groups on day 6, and when the cecal microbiota was analyzed on day 7, there were significant differences between all groups (Supplemental Figure 2A). When the beta diversity was analyzed using non metric multi-dimensional scaling analysis (NMDS), there were significant differences between the groups on days 2, 4, 6 and 7 indicating that there was a difference between the three groups after challenge with *C. difficile* (Supplemental Figure 2B). When only the two infected groups were analyzed using NMDS, there were significant differences between the WT and Δ*hypD* groups on day 0 and day 7 (Supplemental Figure 2C). On day 0, all three groups had a fecal microbiota dominated by the *Enterococcaceae* (Figure 3A). Day 2 post challenge had the highest relative abundance of *Peptostreptococcaceae,* which is the Family *C. difficile* belongs to, in both WT and Δ*hypD* mice, which was also when the greatest weight loss was observed (Figure 1D). By day 7 post challenge, the *Lachnospiraceae* family made up a significant percentage of the cecal microbiota for all three groups, with the highest abundance being in the Δ*hypD* group at 73%, while the WT group and the cef group had 60% and 43% abundance of *Lachnospiraceae* respectively (Figure 3A). Differential abundance analysis was calculated between the WT and Δ*hypD* groups using ALDEx2[45]. For each amplicon sequence variant (ASV) analyzed, ALDEx2 estimates the difference in the centered-log-ratio (a measure of relative abundance) between groups and reports an effect size. The only day that had significant effect sizes for any ASVs was on day 7, when the cecal microbiota was analyzed. Although only 3 ASVs were significant, the top 8 ASVs driving differences between the WT and Δ*hypD* microbiotas were examined via NCBI BLAST to determine the identity of each ASV (Figure 3B). *Lachnospiraceae bacterium* strain D2 1X 41 and *Clostridium* species MD294 were significantly higher in the WT microbiome than the

Δ*hypD* microbiome. *Clostridium* species Clone 49 was significantly higher in the Δ*hypD* microbiota, and there were also two *Lachnospiraceae* strains, including another ASV that resolved to *Lachnospiraceae bacterium* strain D2 1X 41 as well as *Lachnospiraceae bacterium* DW17 that were higher in the Δ*hypD* microbiome, although they did not reach significance. It is unclear why two separate ASVs that resolved to the same strain (*Lachnospiraceae bacterium* strain D2 1X 41) showed such different results in terms of abundance in the WT and Δ*hypD* microbiomes. Of the top 8 ASVs driving the difference between the WT and Δ*hypD* cecal microbiomes on day 7 post challenge, 5 were either *Clostridium* species or members of the *Lachnospiraceae* family, including all statistically significant ASVs (Figure 3B). This suggests that hydroxyproline may be differentially abundant between the two groups of mice and that members of the *Lachnospiraceae* family and the *Clostridium* genus respond to this. Given the previous work showing that commensal *Clostridia* are important to colonization resistance against *C. difficile,* we next wanted to investigate the response of commensal *Clostridia* to hydroxyproline[16, 19].

Hydroxyproline is utilized by commensal *Clostridia* and *C. difficile* when supplemented into a rich media. To test for the utilization of hydroxyproline, WT *C. difficile,* Δ*hypD*, and the commensals *C. hiranonis, C. hylemonae* and *C. scindens* were grown in BHI and in BHI +600mg/L of hydroxyproline (BHI and BHI +hyp) for 14 hr, then amino acids and 5-amino-valerate, the product of proline fermentation, were quantified using LC/MS. As expected, the BHI +hyp control had significantly higher levels of hydroxyproline than the BHI alone (Figure 4A, $p < 0.01$, Student's T Test with Welch's Correction). WT *C. difficile* utilized hydroxyproline, as did all of the commensal *Clostridia*, however, the Δ*hypD* mutant did not (Fig. 4A, $p < 0.01$, Student's T Test with Welch's Correction). There were no significant differences in levels of proline between the

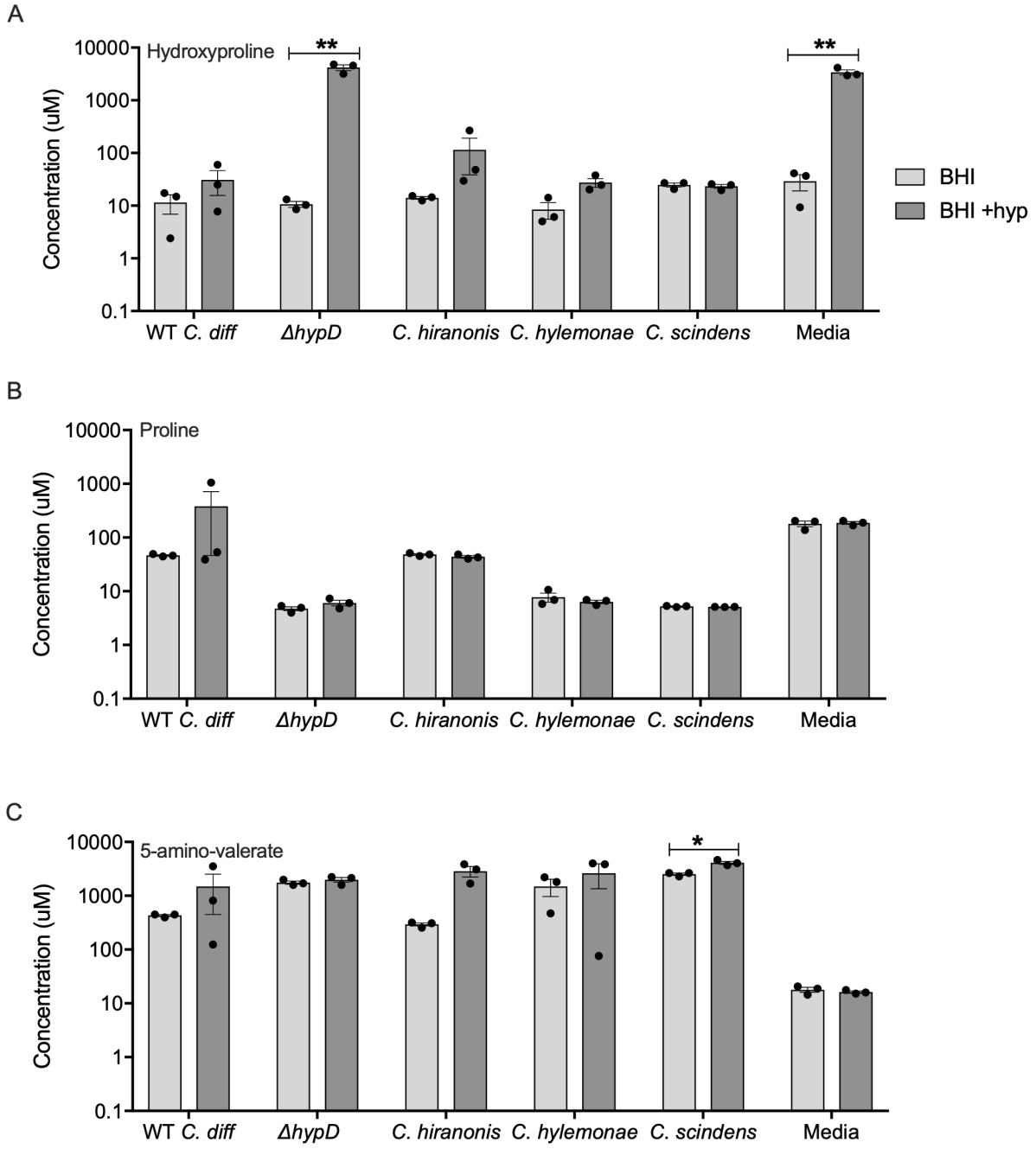

Figure 4: *C. difficile* WT, *C. hiranonis, C. hylemonae,* and *C. scindens* utilize hydroxyproline when it is supplemented into a rich growth medium. Concentration of (**A**) hydroxyproline, (**B**) proline, and (**C**) 5-amino-valerate in BHI and in BHI +600 mg/L hydroxyproline. Supernatants were taken after 24 hours of growth by WT, *hypD, C. hiranonis, C. hylemonae,* or *C. scindens.* BHI alone and BHI +600mg/L of hydroxyproline were used as controls. Statistical significance was determined using Student's T Test with Welch's Correction to account for multiple comparisons (*, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001).

BHI and BHI +hyp, which is likely explained by the fact that the bacteria were grown in a rich media (Figure 4B). The levels of 5-amino-valerate were higher in supernatants for all bacteria grown in BHI or in BHI +hyp than they were in the media control for either condition, indicating that all strains tested were utilizing proline and producing 5-amino-valerate (Figure 4C). Interestingly, the levels of 5-amino-valerate were significantly higher for *C. scindens* in BHI +hyp, although it is unclear as to whether there was enough of a difference in the concentrations to be biologically relevant ($p < 0.01$, Student's T Test with Welch's Correction).

The genomic position of *p5cR* in relation to *hypD* and the transcriptional response to hydroxyproline varies between *C. difficile* and commensal *Clostridia.* To elucidate the greater transcriptional response to hydroxyproline, *C. difficile, C. hiranonis, C. hylemonae* and *C. scindens* were each grown in BHI or BHI + hyp and the relative copy number of *hypD* and *p5cR* was analyzed using qRT-PCR (Figure 5B-E). When *hypD* and *p5cR* were aligned across strains using *C. difficile* as the reference strain, it was found that only *C. difficile* and *C. hiranonis* had the *p5cR* gene next to the *hypD* gene (Figure 5A). In *C. hylemonae* and *C. scindens,* the *p5cR* gene was not adjacent to the *hypD* gene. In addition, *C. hiranonis* showed the greatest percentage of amino acid similarity (84%) to the *C. difficile hypD* gene, while the other two commensals only showed 55% AA similarity (Figure 5A). The percent similarity for *p5cR* was the same for all commensals, as they all showed a 69% AA similarity to the *C. difficile p5cR* gene.

All four strains tested had different responses to hydroxyproline supplementation of rich media (Figure 5B-E). *C. hiranonis* had significantly increased expression of *hypD* and *p5cR* in the presence of hydroxyproline, which was expected given that the two genes are possibly operonic in that strain ($p < 0.001$, Student's T test). *C. hylemonae* had significantly increased expression of *hypD,* but not of *p5cR* ($p < 0.01$, Student's T test). Neither *C. difficile* nor *C. scindens* showed

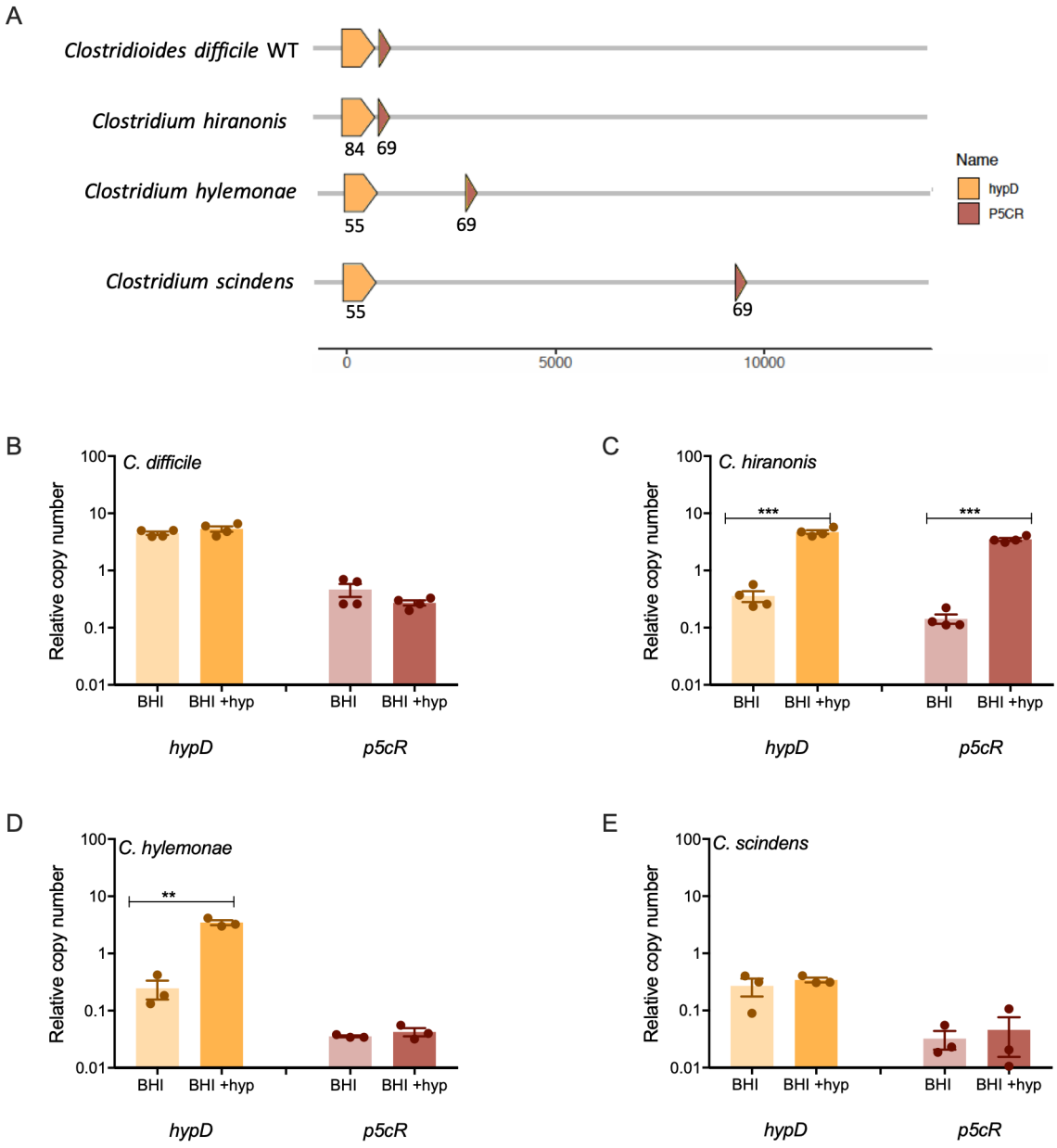

Figure 5: Expression of *hypD* and *p5cR* differs between *C. difficile* and selected commensal *Clostridium* in rich media supplemented with hydroxyproline. **A.** Alignment of *hypD* and *p5cR* across *C. difficile* and selected commensal *Clostridium* strains. Each protein sequence was compared against its counterpart in the reference strain *C. difficile* 630Δ*erm*, generating the amino acid percent identity labeled within each gene. **B-E**. Expression of *hypD* and *p5cR* in BHI media and BHI media with 600 mg/L hydroxyproline added of *C. difficile* (**B**), *C. hiranonis* (**C**), *C. hylemonae* (**D**) and *C. scindens* (**E**). Experiments were run in triplicate and three biological replicates were performed. The expression in medium supplemented with hydroxyproline was compared to expression in medium without additional hydroxyproline. Statistical significance was determined using Student's T Test (*, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001).

significantly increased expression for either gene, but the overall relative copy number for *C. difficile* was approximately ten-fold higher than the relative copy number for *C. scindens* (Figure 5B, 5E).

***C. difficile* and commensal *Clostridia* each have different transcriptomic responses to the presence of hydroxyproline.** *C. difficile*, *C. hiranonis, C. hylemonae* and *C. scindens* were all grown in BHI or BHI supplemented with 600mg/L of hydroxyproline (BHI + hyp) media. At mid-log growth (OD_{600} 0.3-0.5), RNA was extracted, and the transcriptomic response was analyzed using RNAseq. For *C. difficile,* many of the genes that were upregulated upon exposure to hydroxyproline are involved in proline metabolism, including the copy of *proC* that is adjacent to *hypD*. In particular, many of the genes in the *prd* operon, which encodes enzymes for the reduction of proline in Stickland fermentation and has previously been shown to be upregulated in the presence of proline, were upregulated in *C. difficile* (Figure 6A, Supplemental Figure 5A)[26]. Genes involved in regenerating NAD+ via the reduction of succinate and its conversion to butyrate were decreased in expression in the presence of hydroxyproline, consistent with the role of proline reductase as a preferred mechanism of reducing equivalent regeneration. In *C. hiranonis*, most of the differentially expressed genes were downregulated, including amino acid and branched chain amino acid biosynthetic genes, as well as carbohydrate utilization genes. The putative ferrous iron importer gene*feoB2* was increased in *C. hiranonis* in the presence of hydroxyproline, as well as gene encoding a putative NADP-dependent α-hydroxysteroid dehydrogenase, although the overall expression of the latter was quite low (Fig. 6B). Similarly, *C. hylemonae* had several transcripts that significantly decreased with supplementation of hydroxyproline, including those encoding the glycine reductase (Fig. 6C). *C. scindens* had the largest number of differentially expressed genes between the two media conditions (Figure 6D). A number of genes from the *prd* operon were

upregulated in response to hydroxyproline, as were a number of genes encoding subunits of an electron transport complex (*rnfABCDGE*) (Figure 6D, Supplemental Figure 5D). Several genes from the *bai* (bile acid inducible) operon were significantly decreased, although their expression levels in BHI alone were quite low. The expression of genes in the *bai* operon was decreased in *C. scindens* and *C. hylemonae,* in the presence of hydroxyproline but in *C. hiranonis,* the expression of *bai* operon genes was increased in the presence of hydroxyproline (Supplemental Figure 4). Overall, the variable transcriptional responses to the presence of hydroxyproline observed between *C. difficile* and the three commensal *Clostridia* revealed changes in non-hydroxyproline associated metabolic pathways, including those for fermentation of other Stickland substrates, such as the *prd* and *grd* operons (Supplemental Figure 5).

Discussion

Understanding which nutrients are required for *C. difficile* to persist and cause disease in the host is important to developing targeted therapeutics against CDI. In this study, we employed bacterial genetics to examine how the utilization of hydroxyproline by *C. difficile* affects CDI and the microbiome in a mouse model of infection. To facilitate the interpretation of the *in vivo* data, the Δ*hypD* and Δ*p5cr* mutants and their complements were first grown in a minimal medium with hydroxyproline substituted for proline (CDMM –pro). While there was a growth defect in the Δ*hypD* mutant, as expected, no growth defect was observed for the Δ*p5cr* mutant (Figure 1B). This is potentially due to functional redundancy within the *C. difficile630Δerm* genome where a second homolog of P5CR is present (encoded by *CD630_14950*, Supplemental File 1), which did

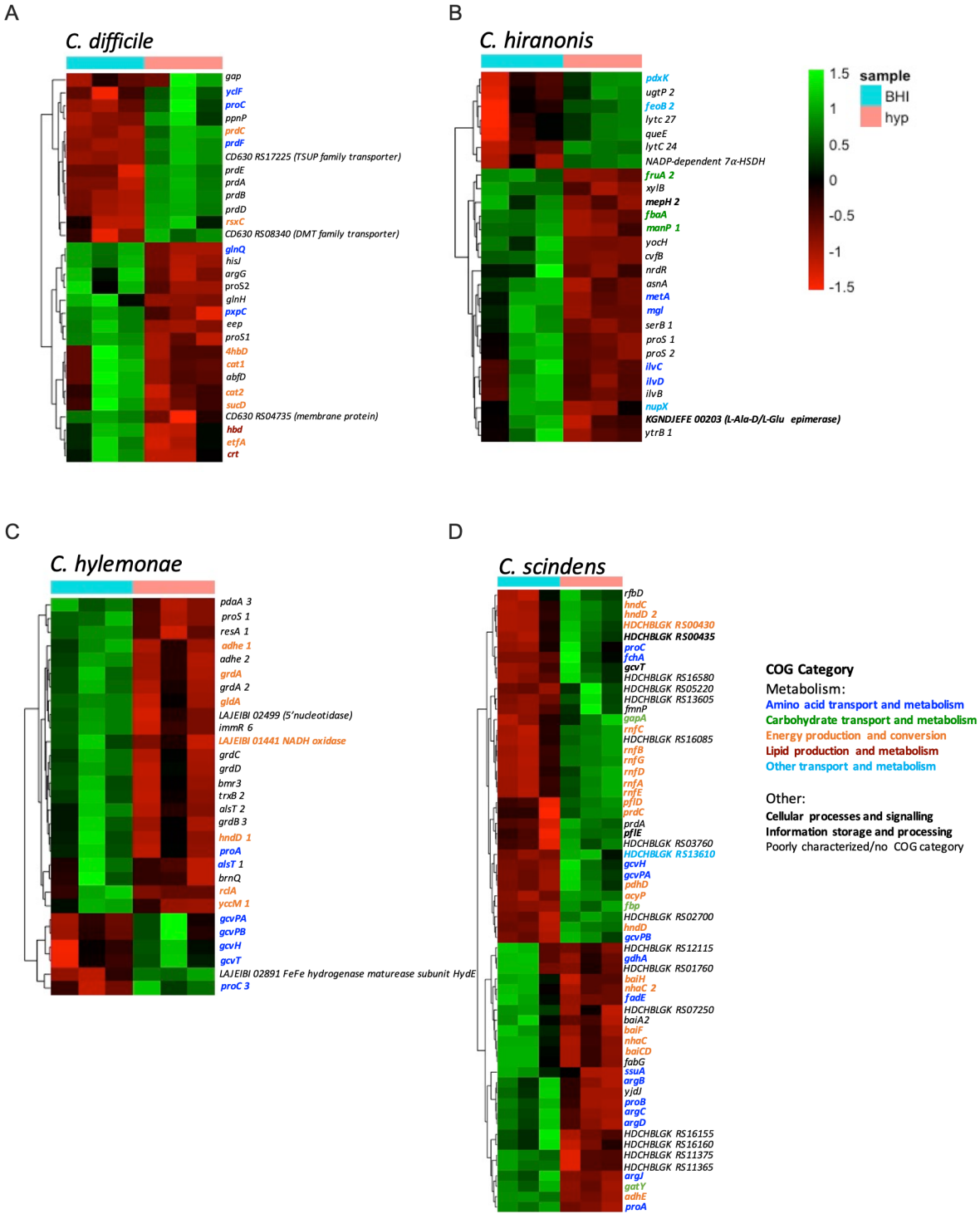

Figure 6: Transcriptomic differences in response to hydroxyproline vary between *C. difficile* and commensal *Clostridia.* Heatmap of genes that had significantly differential expression in **(A)** *C. difficile* WT, **(B)** *C. hiranonis*, **(C)** *C. hylemonae* and **(D)** *C. scindens* between Analysis was run using Geneious and DESeq2. All genes considered differentially expressed had an adjusted p value of <0.05 and ±1 log fold change.

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not significantly change expression in the presence of hydroxyproline. Also of interest is that all strains other than the Δ*hypD* mutant showed significantly increased growth when hydroxyproline was present in the media as opposed to proline. This is likely due to the repression of alternative NAD^+ regeneration pathways that would result in butyrate production, which would impede growth of *C. difficile*, but further experiments are required to test this hypothesis[25, 29, 48].

Since Δ*hypD* growth was impaired *in vitro* when hydroxyproline was the only proline source, we reasoned that *hypD* may be important for colonization and disease progression in a mouse model of CDI. While the fecal burden of *C. difficile* was similar between the strains, the mice challenged with the WT strain showed more weight loss on days 3, 5 and 7 after challenge and toxin activity was higher on day 3 post challenge relative to mice colonized with Δ*hypD* (Figure 2D-E). There was no significant difference in toxin activity 7 days post challenge, indicating that this effect is the strongest earlier during disease. While the effect is subtle, the differences in weight and toxin activity suggest that *C. difficile* relies on hydroxyproline for maximal fitness *in vivo*, highlighting the importance of a host-derived amino acid that is likely made available via toxin-induced expression of host matrix metalloproteinases[14, 49, 50]. *C. difficile* 630 was chosen for this experiment due to the genetic tools available for this strain, but it causes less severe disease in a mouse model than strains R20291 or VPI 10463 [32, 51, 52]. It is possible that a stronger difference between the mutant and the wild type strain would be observed in these strain backgrounds, especially given their increased expression of *hypD* in the presence of hydroxyproline in a defined medium when compared to *C. difficile* 630 (Supplemental Figure 3).

Each of the bacteria tested had a different transcriptional response to hydroxyproline, both in terms of RNAseq and when *hypD* and *p5cr* were tested individually using qRT-PCR (Figures 5-6). Of particular interest was the fact that neither *C. difficile* nor *C. scindens* showed upregulation

of *hypD* or *p5cr* when hydroxyproline was supplemented to the media but when the levels of amino acids were quantified using LC/MS, both organisms metabolized the majority of hydroxyproline present (Figure 4A). For *C. scindens,* this may mean that *hypD* and/or *p5cr* are always transcriptionally active or that the bacterium has another way to utilize hydroxyproline that doesn't require either gene. For *C. difficile* 630Δ*erm,* it is more likely that *hypD* is always transcriptionally active, as *C. difficile* Δ*hypD* did not utilize the excess hydroxyproline added to the media, in addition to the growth defect previously observed (Figure 1B and 4A). The lack of differential expression in *C. difficile* 630 is particularly interesting, as when the *C. difficile* strains 630, R20291 and VPI 10463 were tested in a minimal medium, 630 was the only one where *hypD* was not strongly upregulated in the presence of hydroxyproline, indicating that there are regulatory differences between strains (Supplemental Figure 3). Unfortunately, one of the limitations of the *in vitro* work in this study was the requirement to use a rich and undefined medium, that contains a basal level of hydroxyproline, as *C. hiranonis* and *C. hylemonae* do not grow well in defined media[16, 53].

The overall transcriptional response of *C. difficile* 630Δ*erm* and the commensal *Clostridia* to hydroxyproline indicated *in vitro* that while there were some similarities, each organism had a relatively unique response. In *C. scindens*, over 60 transcripts were significantly altered in response to hydroxyproline, with 38 of those genes being in a metabolic COG category. Of particular interest is that *baiA2, baiCD, baiF and baiH* were all downregulated in response to hydroxyproline, indicating that even without cholate in the media, the activation of the *bai* operon can vary depending on the nutritional content of the media. While none of the changes in *bai* transcripts in *C. hylemonae* were statistically significant, several *bai* operon genes, including *baiG* and *baiE*, were downregulated in response to hydroxyproline (Supplemental File 1). This is

particularly interesting given the previous finding that *C. hylemonae* shows upregulation of the *bai* operon when exposed to cholate in a defined medium, but not when exposed to cholate in BHI [16, 53]. Further work combining bile acids and hydroxyproline are needed to fully dissect transcriptional networks in these organisms. This supports the finding that each of these commensal *Clostridia* have differing metabolic responses to hydroxyproline, and that further elucidation of their nutrient utilization *in vivo* will be fruitful for identifying possible nutritional overlaps with *C. difficile.* This approach may allow for the development of rationally designed cocktails of commensal microbiota that can compete against *C. difficile* for one or more nutrient sources in an infected host.

Acknowledgements

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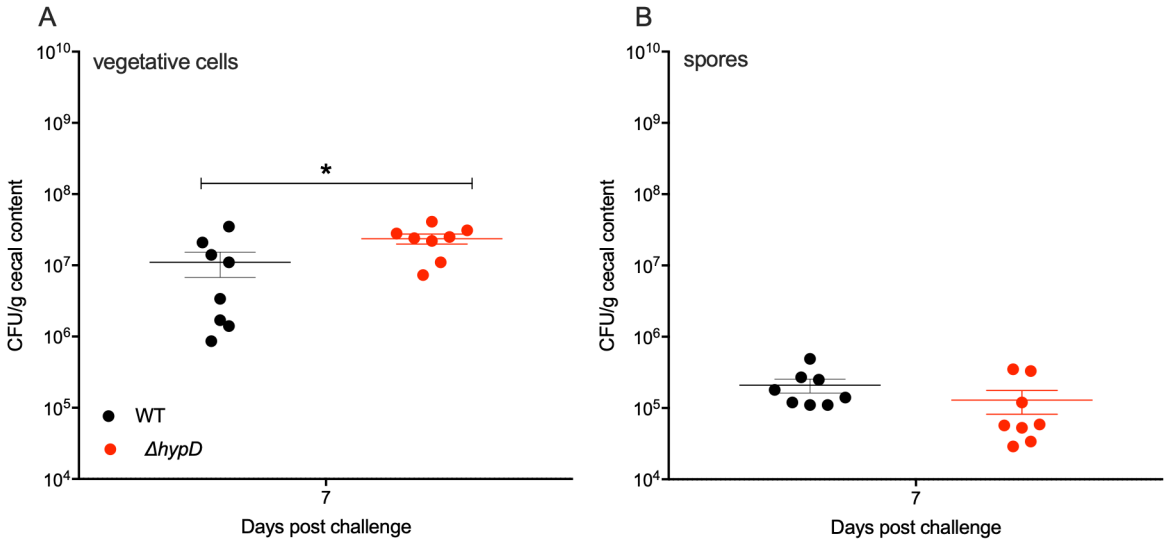
Supplemental Data

Table 1: PCR for deleted genes in C. difficile 630Δerm mutant clones

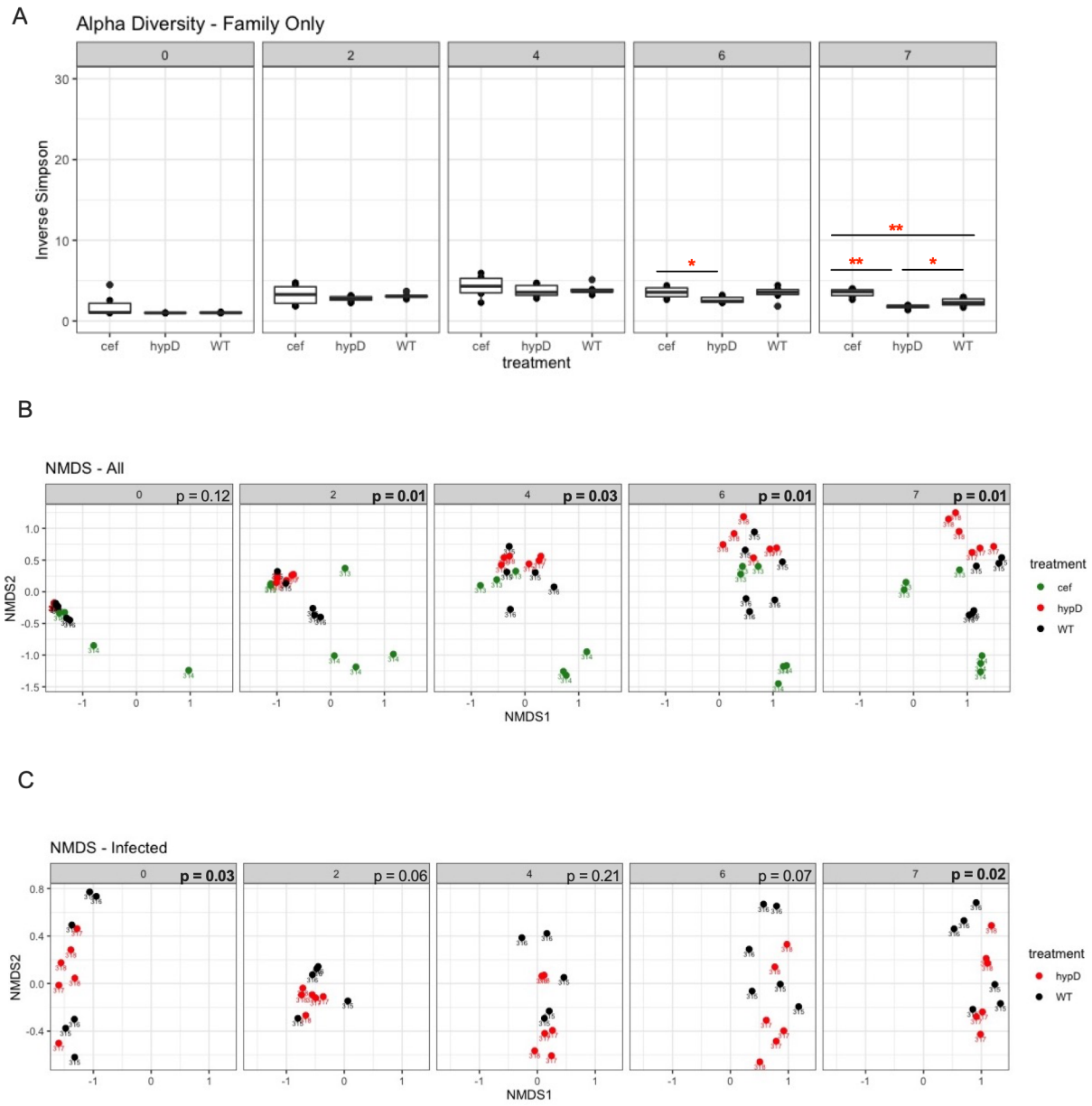
YH-P295	GACAAAATAATAACAAATATAGTATTATGTAATTG	HypD KO in C.diff 630Derm seq primer - Fwd	WT = 4.5kb, KO = 2.2kb, Ta = 60C
YH-P296	CAGCTTCTATAGTAGTTCCTGCTG	HypD KO in C.diff 630Derm seq primer - Rev	WT = 4.5kb, KO = 2.2kb, Ta = 60C
YH-P297	AACACTAGATATATACAAGGTGTTGG	P5CR KO in C.diff 630Derm seq primer - Fwd	WT = 2.9kb, KO = 2.2kb, Ta = 58C
YH-P298	CTTACACTATATTTGTATATTATCTTGGC	P5CR KO in C.diff 630Derm seq primer - Rev	WT = 2.9kb, KO = 2.2kb, Ta = 58C
YH-P299	CTTTTCCAAGTTTCATAGCTATATC	U32-1 KO in C.diff 630Derm seq primer - Fwd	WT = 3.55kb, KO = 2.4kb, Ta = 59C
YH-P300	GAGAAAGAAAATTGTAACATATGATG	U32-1 KO in C.diff 630Derm seq primer - Rev	WT = 3.55kb, KO = 2.4kb, Ta = 59C
YH-P301	ACCTTCCCACATATTTAAATCG	U32-2 KO in C.diff 630Derm seq primer - Fwd	WT = 4.75kb, KO = 2.2kb, Ta = 59C
YH-P302	TTATTATTTGATTCCTATTTTGGC	U32-2 KO in C.diff 630Derm seq primer - Rev	WT = 4.75kb, KO = 2.2kb, Ta = 59C

Table 2: qRT-PCR Primers

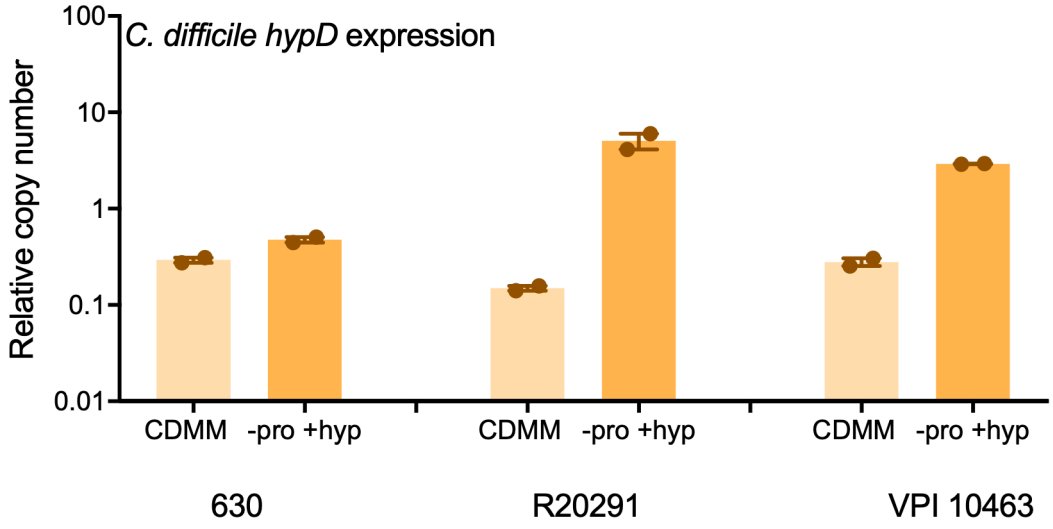
Gene	Forward	Reverse
630-hypD	GCA AGA CAA ATG GCA GAA GAA G	CTG GTT TGT GAG CTG GTA CA
630-p5cR	GGG AAG GAA TGT CAG CTC TTT	TGA TAC TAT CTC TGC CTC ACC A
Chir-hypD	GGT GAA GTT ATG GGT GCT AGT C	GCT GTT GGT CCG TTA GTA TCT
Chir-p5cR	CCA AGA CCT CAG GCT TAC AAA	AGC TGG AGA GCA AAC CAT ATC
Chyl-hypD	CTA CAC TCG GTG TGA ACT TCT G	CGG ATA CAA ACG GTC CTA CAT C
Chyl-p5cR	CCG AAC CTT CTG CAG TTC TT	GAA GTA TGG CGC AGG CTA TT
Csci-hypD	CTG GAA TGC CTG TGG GTA AA	GGC GAA ATC CGT ATA GGT ACT G
Csci-p5cR	TCA GGT GAC AGA CAG CAA TAA G	GCC TGG AGC GAT CGT AAT AAT



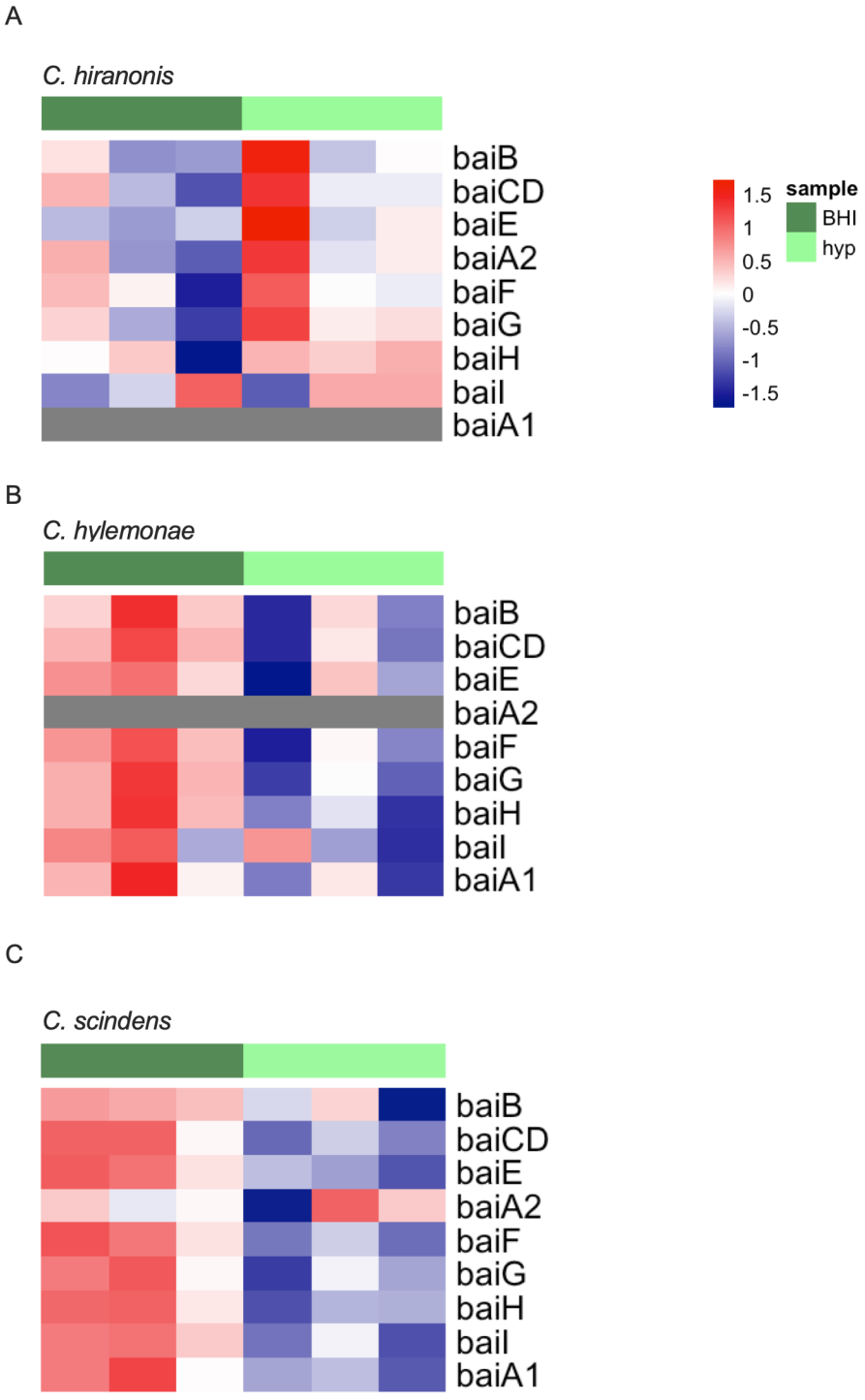
Supplemental Figure 1: Vegetative bacterial load in cecal content is higher in Δ*hypD* mutant than in WT on Day 7. *C. difficile* vegetative cell (**A**) or spore (**B**) CFUs in cecal content on day 7 post challenge. Statistical significance was determined using Mann-Whitney (*, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001).



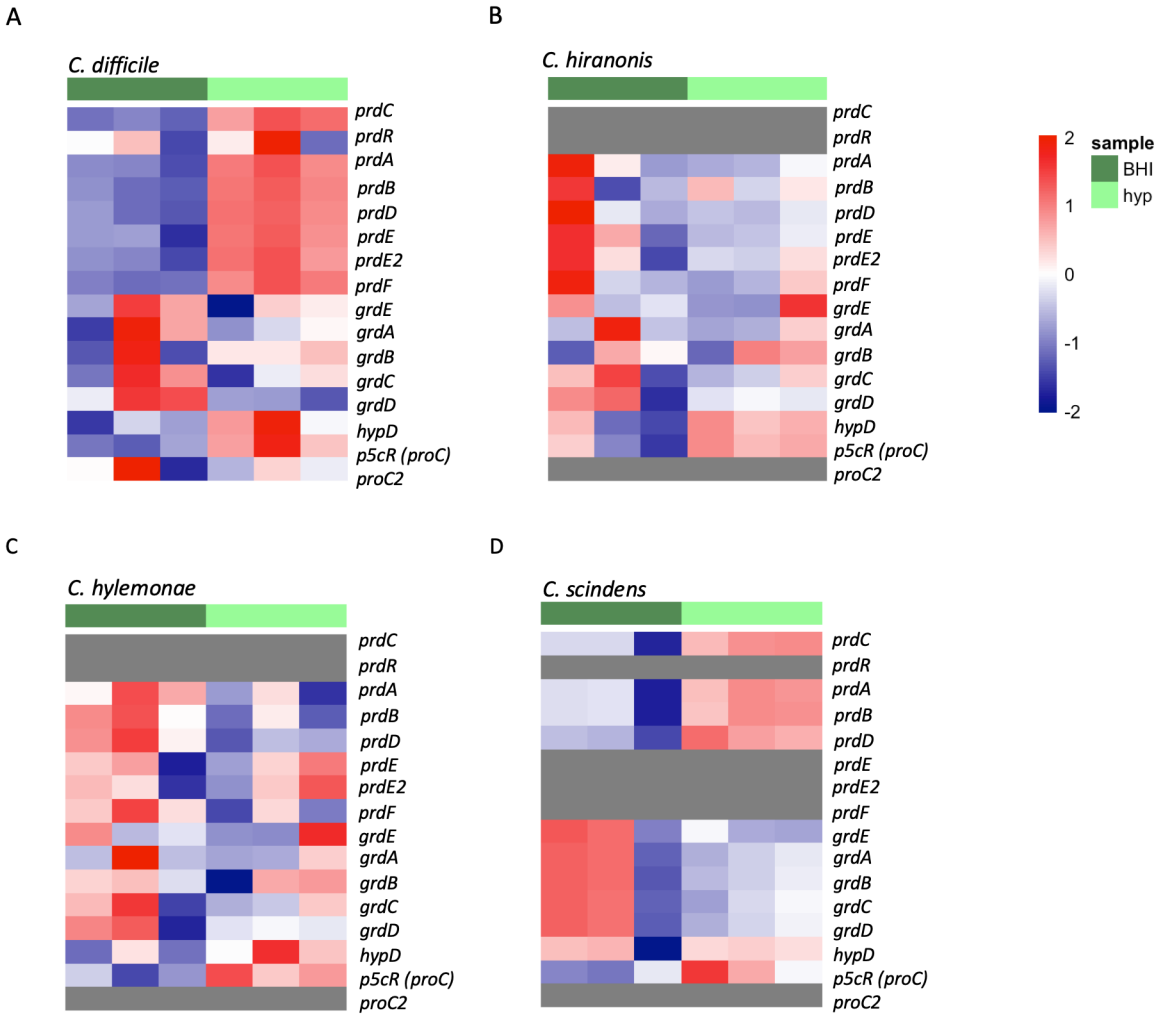
Supplemental Figure 2: The alpha and beta diversity differ between groups on day 7. A. Alpha diversity calculated using inverse simpson at the family level for cef, *hypD* and WT fecal microbiome for days 0, 2, 4, and 6 post challenge and the cecal microbiome for day 7 post challenge. **B.** Beta diversity calculated using NMDS for cef, *hypD* and WT fecal microbiome for days 0, 2, 4, and 6 post challenge and the cecal microbiome for day 7 post challenge. **C.** Beta diversity calculated using NMDS for *hypD* and WT fecal microbiome for days 0, 2, 4, and 6 post challenge and the cecal microbiome for day 7 post challenge. Statistical significance was determined using Kruskal-Wallis for alpha diversity and Permanova Adonis for beta diversity (*, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001).



Supplemental Figure 3. The transcriptional response of *hypD* to hydroxyproline differs between *C. difficile* strains. Expression of *hypD* in CDMM and CDMM –pro +hyp of *C. difficile* 630, R20291 and VPI 10463. Experiments were run in triplicate and two biological replicates were performed.



Supplemental Figure 4: Transcriptional response of *bai* operon to hydroxyproline differs between *C. hiranonis* and other commensal *Clostridia.* Heatmap of *baiA1* and genes within the *bai* operon in BHI and BHI + hyp in **(A)** *C. hiranonis,* **(B)** *C. hylemonae* and **(C)** *C. scindens.*



Supplemental Figure 5: *C. scindens* shows differential regulation of the *prd* and *grd* operon in response to hydroxyproline. Heatmap of genes in the *prd* operon, *grd* operon as well as *hypD, p5cR,* and *proC2* in **(A)** *C. difficile* WT, **(B)** *C. hiranonis*, **(C)** *C. hylemonae* and **(D)** *C. scindens* in BHI alone or in BHI supplemented with 600 mg/L of hydroxyproline. Analysis was run using Geneious and DESeq2.

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Contributions to the Field

When I began my graduate work in 2016, there was still a lot of information about *Clostridium scindens, Clostridium hylemonae,* and *Clostridium hiranonis* that had yet to be elucidated. While it was known that all three organisms were capable of producing deoxycholate (DCA) when cholate (CA) was added to the media, the sensitivity of the selected organisms to DCA and CA had yet to be determined[1,2]. It was also unknown whether these organisms were more or less susceptible than *Clostridioides difficile* to DCA and CA. In addition, while it was known that secondary bile acids such as DCA were associated with protection against *C. difficile,* very little was known about the interaction between *C. difficile* and the commensal *Clostridia* capable of producing these secondary bile acids[3,4]. In addition, while *C. scindens* had been shown to provide a potential therapeutic effect against *C. difficile* infection and that effect was theorized to be due to the production of secondary bile acids, there needed to be more mechanistic work done to determine the mechanism behind the effect that the presence of *C. scindens* had on *C. difficile* infection[5]. While much of the work done on these commensal *Clostridia* focused on the inhibitory secondary bile acids they produced, competition for nutrients has been shown to be important in colonization resistance conferred by the gut microbiota[4,6]. However, the potential ability of commensal *Clostridia* to compete against *C. difficile* for nutrients at this time was unknown.

During the course of my graduate research in the Theriot lab, I was able to make great strides towards further characterizing these commensal *Clostridia.* Not only was I able to determine the effect that the production of DCA by these commensals had on the growth of a clinically relevant strain of *C. difficile,* I was able to elucidate the different responses that these organisms had to CA and DCA in a rich media. The differences between the response to CA

supplementation by *C. scindens, C. hylemonae,* and *C. hiranonis* were significant, especially the unexpected lack of transcriptomic response and DCA production by *C. hylemonae.* This particular bit of data, especially when contrasted with transcriptomic data from *C. hylemonae* supplemented with CA in a defined media, not only highlighted the differences between strains but also indicated the potential role of nutrients in the production of DCA and the regulation of the bile acid inducible (*bai*) operon by these commensals[7].

During the work covered in the third chapter of my dissertation, I was able to further investigate the reaction of these commensals to nutrient availability, specifically hydroxyproline supplementation. Not only did I examine the effect of the ability to utilize hydroxyproline on *C. difficile* infection, but I also discovered that *C. difficile, C. scindens, C. hiranonis* and *C. hylemonae* had very different transcriptional responses to hydroxyproline supplementation. In the commensal *Clostridia* tested, hydroxyproline supplementation suppressed transcription of genes in the *bai* operon. This further advances the theory that the regulation of the *bai* operon is related to nutrient availability, and opens up many further avenues of investigation. The work I have done with these commensal *Clostridia* has moved us closer to being able to rationally manipulate the microbiota in the context of *C. difficile* infection.

In the future, it will be important to continue investigating the role of bile acid production by these commensal *Clostridia in vivo,* as well as the impact that the availability of primary bile acids such as CA has on the microbiota as a whole. In addition, further work on elucidating the regulation of the *bai* operon is needed, as well as investigation of the effect that various nutritional landscapes have on these commensal *Clostridia* and their ability to compete against *C. difficile.* While the work that I and others have done during these past five years have significantly moved this field forward, there is always more to do.

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