The isolation of novel *Lachnospiraceae* strains and the evaluation of their potential roles in colonization resistance against *Clostridium difficile*

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Abstract

Background: Antibiotic disruption of the gastrointestinal tract's indigenous microbiota can lead to one of the most common nosocomial infections, *Clostridium difficile*, which has an annual cost exceeding $4.8 billion dollars. The gut's unperturbed microbiota endow a protective effect against potential pathogens, inducing colonization resistance. Recent studies have found that mice resistant to *C. difficile* colonization, compared to those susceptible to *C. difficile*, have higher levels of microbes in the *Firmicutes* phylum, especially those of the *Lachnospiraceae* family. Another study showed that an isolate, *Lachnospiraceae* D4, which is closely related to *Clostridium clostridioforme* in 16S rRNA gene sequence, was able to restore partial colonization resistance to *C. difficile* in a germfree mouse. This study evaluates whether 23 *Lachnospiraceae* isolates can directly inhibit *C. difficile* and whether they have bile salt hydrolase and/or 7α-dehydroxylase activity, which may affect *C. difficile* germination and growth.

Methods: *Lachnospiraceae* isolates were obtained from C57BL/6 mouse cecal content, cecal tissue and feces then screened via a plate wash PCR procedure using *Lachnospiraceae*-specific primers. Direct inhibition of *C. difficile* by *Lachnospiraceae* isolates and communities was evaluated using an overlay plate technique. The presence of bile salt hydrolase was assessed *in vitro* using a plate assay and the presence 7α-dehydroxylase activity was assessed by PCR with *baiCD*-specific primers.

Results: We successfully isolated 20 *Lachnospiraceae* isolates. Together with 3 *Lachnospiraceae* isolates, including *Lachnospiraceae* D4, from Reeves, et al. none were able to directly inhibit *C. difficile* growth either through cell cultures or supernatants. However, we found 3 *Lachnospiraceae* isolates with bile salt hydrolase activity and 4 with 7α-dehydroxylase activity.

Conclusions: If *Lachnospiraceae* are able to resist *C. difficile* colonization in the gastrointestinal tract, it is unlikely through direct inhibition. It is possible that by participating in varying points in the pathway of converting conjugated primary bile acids to secondary primary bile acids, a community of *Lachnospiraceae* can work together to restore colonization resistance against *C. difficile*. This aids in the development of probiotics against *C. difficile* infection.

Introduction

Clostridium difficile

Clostridium difficile is a Gram-positive, anaerobic, bacteria that is enteropathogenic[5]. Vegetative *C. difficile* cells die rapidly on surfaces, or within six hours on moist surfaces or in the air. *C. difficile* spores can remain viable for months in the environment and can resist hospital disinfectants[10,18]. In hospitals, *C. difficile* spores shed through the gastrointestinal (GI) tract of infected patients can be transmitted to other patients through the hands or equipment of hospital workers or directly from patient to patient[10,17]. Once inside a new host, *C. difficile* spores are able to resist the acidity of the stomach and germinate inside the GI tract[10,17]. In its vegetative state, *C. difficile* cells will produce two toxins, TcdA and TcdB[10,32]. Both toxins are glucosylating toxins, which result in inactivation of GTPases, disassembly of the actin cytoskeleton, and eventually cell death[29]. The cumulative effects of TcdA and TcdB activities in the body will lead to severe inflammation of the colon (colitis), the formation of a pseudomembrane and severe diarrhea[10,32].

C. difficile is the leading cause of nosocomial diarrhea in developed countries[5] and can lead to life-threatening inflammation of the colon[8]. Unfortunately, healthcare costs, number of cases, severity and difficulty of treatment of *C. difficile* have all been increasing in recent years. Data from 2012 shows that the annual cost of *C. difficile* exceeds $4.8 billion dollars[9].

The number of *C. difficile* cases has been increasing since the 1980s[21]. For example, within a six-year period of 2000 to 2005 in the United States, the number of CDI cases admitted into the hospital more than doubled from 139,000 to 301,200[10]. In hospitals, the nosocomial infection rate of CDI has surpassed the infection rate of methicillin-resistant *Staphyloccocus aureus*[27,23]. The phenomenon of increasing CDI cases is paralleled by the emergence of a new "at-risk" population. While those traditionally considered "at-risk" are seniors or hospitalized patients, there have been increasing CDI cases among children and those in community. A study done in Minnesota found that 41% of CDI cases were community-acquired[20] as in the patient had CDI symptoms at least twelve weeks after their most recent hospital discharge. This is an alarming trend considering that, traditionally, most cases of CDI are healthcare-associated in which patients have *C. difficile* symptoms occurring at least two days after admission into a healthcare facility.

As both healthcare-acquired and community-acquired cases of CDI increase, it leaves a significant impact on the costs associated with *C. difficile*. Hospitalized CDI patients will spend, on average, an additional one to three weeks in the hospital than non-CDI hospitalized patients[5]. Yet, the treatment of *C. difficile* is becoming more difficult. For instance, the number of recurrent CDI cases, after initially successful treatments with antibiotics such as metronidazole or vancomycin, is increasing[7]. Currently, the mortality rates of CDI show no signs of deceleration[5,21]. From 2000 to 2004, the age-adjusted fatality rate of CDI doubled from 1.2% to 2.2%[10]. Annually, *C. difficile* now causes more than 6,000 deaths in the United States[2,33].

Colonization Resistance

The mammalian GI tract contains the indigenous gut microbiota whose role in health maintenance has been long conceptualized since the early 1900s. It was observed that disturbances, such as those through antibiotics, in the normal enteric microbiota increase the susceptibility of a host to pathogens[7]. These disturbances are elicited through changes in the community structure of the microbiota and in its overall size[6]. Therefore, it was suggested that the complex and undisturbed microbial community has an inhibitory role in the colonization and growth of pathogens. These indigenous microbes of the mammalian gut, by suppressing the colonization and growth of harmful organisms within the host, confer colonization resistance[7].

Antibiotic usage is one of the greatest risk factors behind the development of CDI. Antibiotics alter colonization resistance, leaving the host susceptible to *C. difficile*[5,8,36]. Antibiotics disrupt the protective function of the host's indigenous gut microbiota by disturbing the ecology of the GI tract. A past study found that patients with recurrent antibiotic-associated diarrhea due to *C. difficile* (CDAD) had greater variability of bacterial compositions in their fecal communities and a decrease in the abundance of bacteria of the *Bacteroidetes* and *Firmicutes* phyla[8]. On the other hand, healthy individuals tend to have greater abundances of bacteria of the *Bacteroidetes* and *Firmicutes* phyla[8]. Moreover, patients with recurrent CDI are less likely to have the ability to restore their indigenous GI tract communities. This is further supported by the restoration of colonization resistance in patients after given bacterial communities, via fecal matter transplant[7], from a healthy individual. Despite steps taken to better understand colonization resistance, much remains unknown[36]. Isolating the organisms responsible for colonization resistance, including those of the *Bacteroidetes* and *Firmicutes* phyla, that may be susceptible to disease-rendering

antibiotics and understanding their mechanisms is an important step in alleviating the present *C. difficile* burden.

There are a few hypotheses of the mechanisms behind colonization resistance. One potential mechanism is that regarding bile acids. Bile acids mediate a multitude of functions in human health and, in terms of *C. difficile*, their effects vary as well. Through the conversion of conjugated primary bile acids to secondary bile acids, products of this pathway can either activate *C. difficile* germination or inhibit *C. difficile* growth[7]. As members of the gut microbiota are essential in the bile salt pathway, antibiotic disturbance of key microbes may yield an increase of bile acids that stimulate *C. difficile* germination and/or growth.

Other proposed mechanisms of colonization resistance include the competition for limiting nutrients and/or attachment space. It is possible that the indigenous microbiota outcompete *C. difficile* for those limiting nutrients. Antibiotics that kill these competitively superior microbes provide *C. difficile* access to once-limited nutrients, allowing for its colonization and outgrowth. Additionally, certain indigenous microbes of the GI tract may produce and secrete microbicides against *C. difficile*. If these microbes are removed, then the absence of their microbicides will allow for *C. difficile* growth[7].

Previous work has guided us in the understanding of colonization resistance. It is known that different antibiotics induce varying degrees of *C. difficile* susceptibility in the host and it is plausible that the loss of colonization resistance is not only due to the overall disruption of the indigenous microbiota, but also to the loss of specific members. The loss of these microbes may lead to a loss of direct inhibition, a perturbation of the bile acid pool, a decrease in competitive inhibition of *C. difficile*, etc. Through whichever means, loss of the indigenous gut microbiota and specific members leads to loss of its protective function against *C. difficile*.

Lachnospiraceae

Recent work by Reeves, et al. found that mice that became susceptible and moribund to *C. difficile* after antibiotic application, had increased levels of *Proteobacteria*, and especially those in the family *Enterobacteriaceae*. Contrastingly, healthy mice had *Firmicutes* dominance, with a high prevalence of members of the *Lachnospiraceae* family[25]. Furthermore, in another study by

Reeves, et al. *Lachnospiraceae* D4 (an isolate closely related to *Clostridium clostridioforme* based on 16S rRNA gene sequences) and *Escherichia coli* were used to precolonize germfree mice before *C. difficile* strain VPI 10463 challenge. All mice monocolonized with *C. difficile* were moribund within two days after *C. difficile* challenge. Similarly, all mice precolonized with *E. coli* then challenged with *C. difficile* were moribund within two days of challenge. However, out of the 14 mice precolonized with *Lachnospiraceae* D4 and later challenged with *C. difficile*, only 3 were moribund and 11 were clinically healthy at 2 days post challenge[24]. These studies suggest that members of the *Lachnospiraceae* family are important members in the colonization resistance against *C. difficile*.

Members of the *Lachnospiraceae* family are major constituents of the human GI tract[15,22]. These microbes are strictly anaerobic and are mostly non-spore forming[13,22]. Taxonomic classification of *Lachnospiraceae* members is based only on 16S rRNA-encoding genes[13,22], yielding 24 NCBI-named genera with several that are unclassified[28,22]. Currently, members of *Lachnospiraceae* are known for their abilities to produce butyric acid, a short chain fatty acid, which is associated with obesity[11,22] but to protect against colon cancer[17,22]. However, in terms of colonization resistance, *Lachnospiraceae*'s role remains unknown.

Only a handful of the total number of microbes in the GI tract has been cultured. The rest are yet to be cultured, indicating the lack current investment in isolating potentially important members of the gut microbiome. The cultivation of these microbes endows many benefits that popular molecular-based methods, which focus on genomic sequences, are limited to. For example, microbial cultivation can help reveal the ecological roles of communities in health or disease[1]. Also, many of these uncultured bacteria play important roles in the production, utilization and cycling of nutrients. Consequently, they have a large impact on the other surrounding microbes and the health of the host[31]. Although past work in the Young Lab has demonstrated the importance of *Lachnospiraceae* in the gut microbiota, few members of this family have been cultured.

Objectives

We isolated novel murine *Lachnospiraceae* bacterial strains, which until now have not been cultured, from cecal content, cecal tissue and fecal samples of untreated C57BL/6 mice. The

unperturbed gut microbiota of these mice are likely to have highly represented members of the *Lachnospiraceae* family. We used molecular biology approaches to specifically target and enrich for members of this bacterial family. Each *Lachnospiraceae* isolate was assessed for a direct inhibitory role against *C. difficile* growth and for the presence of bile salt hydrolase activity. *Lachnospiraceae* isolates were also screened for 7α-dehydroxylase, which catalyzes the production of deoxycholate from cholate. Overall, we isolated *Lachnospiraceae* members from a *C. difficile*-resistant murine model and evaluated their potential effects against *C. difficile* colonization through direct inhibition or bile salt mechanisms.

Materials & Methods

Sample collection

Cecal tissue, cecal content and fecal samples were collected from C57BL/6 mice from the University of Michigan Young Lab breeding colony and from The Jackson Laboratory. Mouse ceca were sterilely removed and immediately transferred into an anaerobic chamber (Coy Industries, Grass Lake, MI). Anaerobic 1× phosphate-buffered saline (PBS) was added to the ceca. A sterile scalpel was used to open and separate cecal content and cecal tissue. Cecal tissue was washed with PBS and homogenized. All samples from ceca and feces were diluted, at a 1:10 ratio, to a final dilution of 10^{-9}. Each dilution was plated out in triplicate onto media described below.

Bacterial isolation and selective growth conditions

Dilutions of cecal tissue, cecal content and fecal samples were plated out onto brain heart infusion agar (BD Biosciences) with 0.01% cysteine (BHI). *Lachnospiraceae* were isolated from BHI variations of BHI + 5% fetal bovine serum (FBS); BHI + 10% taurocholate; BHI + 1 μg/mL aztreonam + 10 μg/mL colistin + 2 μg/mL gentamycin; and BHI + 0.5 μg/mL ampicillin + 2 μg/mL erythromycin + 0.25 μg/mL vancomycin. Other *Lachnospiraceae* isolates were obtained from yeast, casitone, fatty acid medium supplemented with glucose (YCFAG)[12] + 2 μg/mL gentamycin + 1 μg/mL aztreonam + 10 μg/mL colistin.

Bacterial isolates were isolated by plate wash technique[30,24]. The plate wash technique is a high throughput method, which allows for simultaneous screening of 94 strains of bacteria. Agar plates with cecal content, cecal tissue and/or feces dilutions, as described above, yielding ~100 colonies were selected. If collection of all colonies and PCR with *Lachnospiraceae*-specific primers (described below) indicated the presence of *Lachnospiraceae*, the procedure was continued. From the original plate's duplicate, each colony was individually cultured and screened with *Lachnospiraceae*-specific primers. Samples with positive hits were saved down. 20% final concentration glycerol stocks of all isolates were created and stored at -80 °C. All bacterial isolation and *C. difficile* work was done in an anaerobic chamber (Coy Industries).

Design of *Lachnospiraceae* 16S rRNA-encoding gene primers

Full length 16S rRNA-encoding genes from 9 abundant *Firmicutes* and *Bacteroidetes* from Berry, et al.[4], 4 *Firmicutes* (*C. difficile*, *Enterococcus faecalis, Lactobacillus murinus*, and *Staphylococcus xylosus*), 6 *Lachnospiraceae* (*Clostridium aldenense*, *Clostridium bolteae*, *Clostridium citroniae*, *Clostridium indolis*, *Clostridium propionicum*, *Clostridium xylosus*), 3 *Lachnospiraceae* phylotypes isolated from the Young Lab (D4, G11, and E7), and 12 of the most represented *Lachnospiraceae*-classified OTUs from Reeves, et al.[25] were aligned. Areas of homology between *Lachnospiraceae* that were not homologous to other *Firmicutes* and *Bacteroidetes* were used to create 5 *Lachnospiraceae*-specific 16S rRNA-encoding forward gene primers: LachF (5' -CC GCA TAA GCG CAC AGC- 3'), Lachno419F (5' –GAC GCC GCG TGA GTG AAG AAG TAT- 3'), Lachno428F (5' –GTA AAG CTC TAT CAG CAG GGA AGA- 3'), Lachno481F (5' –GAC GGT ACC TGA CTA AGA AGC CC- 3'), and Lachno462F (5' –GTC CAC AGG ACT TTG GAC GG- 3'). These forward primers were used with the 16S rRNA-encoding reverse gene primer 1492R (5' -GGT TAC CTT GTT ACG ACT T- 3'). One *Lachnospiraceae*-specific 16S rRNA-encoding reverse gene primer was also created: Lachno1261R (5' -TCG CTT CCC TTT GTT TAC GC- 3'), which was used with the 16S rRNA-encoding forward gene primer 8F (5' -AGA GTT TGA TCC TGG CTC AG- 3'). All primers yield an expected PCR product of approximately 1,320 base pairs.

Gradient PCR was performed to reveal the ideal annealing temperature of 57 °C. All *Lachnospiraceae*-specific primers' specificity and coverage (Table 1) were obtained using the Ribosomal Database Project Probe Match tool. Additionally, primers were tested with Gram-negative bacteria *Bacteroides fragilis Escherichia coli*; Gram-positive bacteria *Lactobacillus murinus*, *Staphylococcus aureus*, *Staphylococcus pneumonia*; DNA from a strain of Ruminococcus; *Clostridium difficile* strain VPI 10463; and Young Lab *Lachnospiraceae* isolates D4, E7 and G3; and a negative control of water.

DNA extraction and 16S ribosomal rRNA-encoding gene sequencing

Genomic bacterial DNA was extracted using the Easy-DNA™ (Invitrogen) kit. Spectrophotometry was performed with Nanodrop 1000 Spectrophotometer (Thermo Scientific). 16S rRNA-encoding genes were amplified using the 8F and 1492R PCR primers with denaturation of 2 min at 94 °C, which was followed by 30 cycles 94 °C for 30 s, 58 °C for 45 s and 72 °C for 90 s. A final extension at 72 °C for 10 min was performed. PCR product cleanup was performed

using the ExoSAP (Affymetrix) protocol. Full-length 16S rRNA amplicons were sequenced at the University of Michigan DNA Sequencing Core using primers 8F (5' -AGA GTT TGA TCC TGG CTC AG- 3'), 515F (5' -GTG CCA GCM GCC GCG GTA- 3'), E939R (5' -CTT GTG CGG GCC CCC GTC AAT TC- 3'), and 1492R (5' -GGT TAC CTT GTT ACG ACT T- 3').

Phylogenetic analyses

The top ten most abundant unclassified *Lachnospiraceae* OTUs of the murine gut microbiota were obtained from Supplementary Figure 4 of Theriot, et al[35]. Partial V3-V5 regions of 16S rRNA-encoding genes of 23 *Lachnospiraceae* isolates and 10 of the most abundant unclassified *Lachnospiraceae* OTUs[35] were aligned using SeqMan (Lasergene). The phylogenetic tree was generated with the Weighbor weighted neighbor-joining tree-building algorithm by the Ribosomal Database Project (Michigan State University) and edited with Mega5[34].

Direct inhibition

Lachnospiraceae isolates were plated onto BHI agar and incubated at 37 °C. At full growth, all colonies were collected and resuspended in brain heart infusion broth (BD Biosciences) + 0.01% cysteine (BHI broth) to obtain turbid cultures. 10 μL of each cell suspension was spotted onto a thinly layered BHI agarose plate and allowed to grow anaerobically for 48 hrs at 37 °C.

Communities of isolates were also tested for direct inhibition of *C. difficile*. All strains isolated from cecal tissue were individually streaked out onto BHI media. At full growth, cultures from each strain were collected and resuspended into its own culture of BHI. 10 μL of liquid culture from each strain was mixed into a community culture (Figure 2), which was spotted onto a thinly layered BHI agarose plate and incubated anaerobically for 48 hrs at 37°C. This was also performed for all strains isolated from cecal tissue and from feces.

Overlay media was prepared with BHI broth + 1% agarose and autoclaved. Anaerobically, a single colony of *Clostridium difficile* strain VPI 10463 was incubated at 37 °C in 5 mL of BHI broth. At full growth, a 1:20 dilution of *C. difficile* culture and 0.01% anaerobic cysteine was added to the overlay media. The overlay media was gently poured over the bottom blot layer and incubated at 37 °C. Twenty-four hours later, direct inhibition of *Clostridium difficile* was assessed.

In order to test for the direct inhibition of *C. difficile* by supernatant, liquid cultures were centrifuged for 10 min. at 6,000 RPM. 10 µL of the supernatant was spotted over the overlay media layer and incubated anaerobically at 37 °C for 24 hours. The direction inhibition by supernatant was then assessed.

Bile salt hydrolase (BSH) detection

The detection of bile salt hydrolase in *Lachnospiraceae* isolates was performed using a plate assay technique[16]. Ten µL of *Lachnospiraceae* isolates, cultured in BHI broth, were spotted onto BHI agar + 0.5% taurocholic acid sodium salt hydrate (Sigma-Aldrich) + 0.5% sodium glycocholate (Chem-Impex International Inc.) + 0.37 g/L $CaCl_2$ (Sigma-Aldrich). Samples were incubated at 37 °C in an anaerobic chamber. Forty-eight hours later, the formation of a precipitate was observed in order to analyze the presence of bile salt hydrolase in each strain. If amino acids are hydrolyzed from their respective bile acids, they will form a solid compound with calcium.

Polymerase chain reaction assay for bile acid 7α-dehydroxylase detection

The presence of the 7α-dehydroxylase enzyme was screened for by amplification of the *baiCD* gene, which is specific to the bile acid 7α-dehydroxylation conversion of cholate to deoxycholate. PCR using degenerate primers baiCD-F (5'-GGWTTCAGCCCRCAGATGTTCTTTG-3V) and baiCD-R (5V- GAATTCCGGGTTCATGAACATTCTKCKAAG- 3V) from Wells, et al.[37] were performed on each *Lachnospiraceae* extracted DNA sample using the illustra™ PuReTaq™ Ready-To-Go™ PCR beads (GE Healthcare) with 100 ng of template DNA, 0.5 µM/L of each primer, and water to a total volume of 25 µL. Amplification of the samples was completed in a DNA thermal cycler (Eppendorf Mastercycler gradient). Denaturation occurred for 2 min at 94 °C and was followed by 35 cycles of 20 s at 94 °C, 30 s at 52 °C and 90s at 69 °C. A final extension for 10 min at 68 °C was performed. A negative control of sterile water and a positive control of *Clostridium scindens* strain VPI 12708 were used. PCR products were separated with a 1% agarose gel electrophoresis and viewed under UV excitation. Bands were compared to a 1 Kb Plus DNA Ladder (Invitrogen).

Primer	Specificity to *Lachnospiraceae*	% *Lachnospiraceae* coverage
LachF	98.1%	9.7%
Lachno 419F	94.2%	24.8%
Lachno428F	96.8%	57.2%
Lachno462F	100%	0.004%
Lachno481F	97.1%	45.5%
Lachno1261R	96.8%	21.9%

Table 1. Six *Lachnospiraceae*-specific primers were used to isolate *Lachnospiraceae* with the plate wash polymerase chain reaction technique. % Specificity is the ratio of *Lachnospiraceae* primer matches to the total number of primer matches while % coverage is the ratio of primer matches to *Lachnospiraceae* to the total number of members in the *Lachnospiraceae* family.

Results

Isolation of novel *Lachnospiraceae* strains

From untreated C57BL/6 mice cecal content, cecal tissue and feces, we targeted *Lachnospiraceae* microbes. We used *Lachnospiraceae*-specific primers for 16S rRNA-encoding genes and a plate wash PCR technique[30] utilizing specific media, which enrich for *Lachnospiraceae*. Out of those tested, the greatest *Lachnospiraceae* enrichment media was BHI with 1 μg/mL aztreonam, 2 μg/mL gentamycin and 10 μg/mL colistin under anaerobic conditions.

Out of 45 unique isolates from a C57BL/6 murine mouse, isolated with *Lachnospiraceae*-specific 16s rRNA-encoding gene primers through the plate wash PCR technique, 20 were previously uncultured *Lachnospiraceae* strains (Table 2). While BHI is a sufficient enrichment media for 17 of the 20 isolates, 3 necessitated the addition of 5% fetal bovine serum (FBS) for adequate growth conditions in an anaerobic environment. Of the isolates, all grew poorly in broth culture. On solid media, 11 isolates took 5+ days, 4 took 4 days, 4 took 3 days and 1 took 1 day to reach full growth. All 20 isolated *Lachnospiraceae* strains and 3 *Lachnospiraceae* strains from Reeves, et al.[24] were used to further tested for the ability to inhibit *C. difficile* growth and to convert bile acids.

Full-length chromosomal DNA of *Lachnospiraceae* isolates was extracted and 16S rRNA-encoding DNA was amplified and sequenced. The V3-V5 regions of 16S rRNA-encoding gene regions were aligned to corresponding regions of the top 10 most abundant *Lachnospiraceae* OTUs from Theriot, et al.[35] A phylogenetic tree was generated using the neighbor-joining method with *Escherichia coli* as the outgroup (Figure 1). Phylogenetic analysis of these 23 *Lachnospiraceae* isolates with the top 10 most abundant *Lachnospiraceae* OTUs from Theriot, et al.[35] indicates that the diversity of our isolates substantially overlaps that of the 10 most abundant *Lachnospiraceae* OTUs. Comparisons of V3-V5 regions of the 16s rRNA-encoding genes reveal that some *Lachnospiraceae* isolates have a high percentage of nucleotide similarity amongst each other. This may imply that if abundant *Lachnospiraceae* of the murine GI tract play a significant role in colonization resistance, closely related *Lachnospiraceae* isolates may function in a similar responsibility. However, it is also possible that physiological roles of individual species vary across a phylogenetic classification in that, within the family of *Lachnospiraceae*, only a few members of those tested confer a given characteristic.

Isolate	RDP Classification
DW 3	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; unclassified_Lachnospiraceae
DW 7	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; unclassified_Lachnospiraceae
DW 8	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; unclassified_Lachnospiraceae
DW 11	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; unclassified_Lachnospiraceae
DW 12	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; unclassified_Lachnospiraceae
DW 17	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; unclassified_Lachnospiraceae
DW 21	Firmicutes; Clostridia; Clostridiales; unclassified_Clostridiales
DW 22	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; unclassified_Lachnospiraceae
DW 28	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Blautia
DW 34	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; unclassified_Lachnospiraceae
DW 42	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Clostridium XlVb
DW 44	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; unclassified_Lachnospiraceae
DW 46	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; unclassified_Lachnospiraceae
DW 52	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; unclassified_Lachnospiraceae
DW 59	Firmicutes; Clostridia; Clostridiales; unclassified_Clostridiales
DW 60	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; unclassified_Lachnospiraceae
DW 61	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; unclassified_Lachnospiraceae
DW 67	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; unclassified_Lachnospiraceae
DW 68	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; unclassified_Lachnospiraceae
DW 70	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; unclassified_Lachnospiraceae
D4	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Clostridium XlVa
E7	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; unclassified_Lachnospiraceae
G11	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; unclassified_Lachnospiraceae

Table 2. Twenty-three *Lachnospiraceae* isolates and their Ribosomal Database Project (RDP) classification. Three *Lachnospiraceae* (in the shaded boxes) are from Reeves, et al.[24]

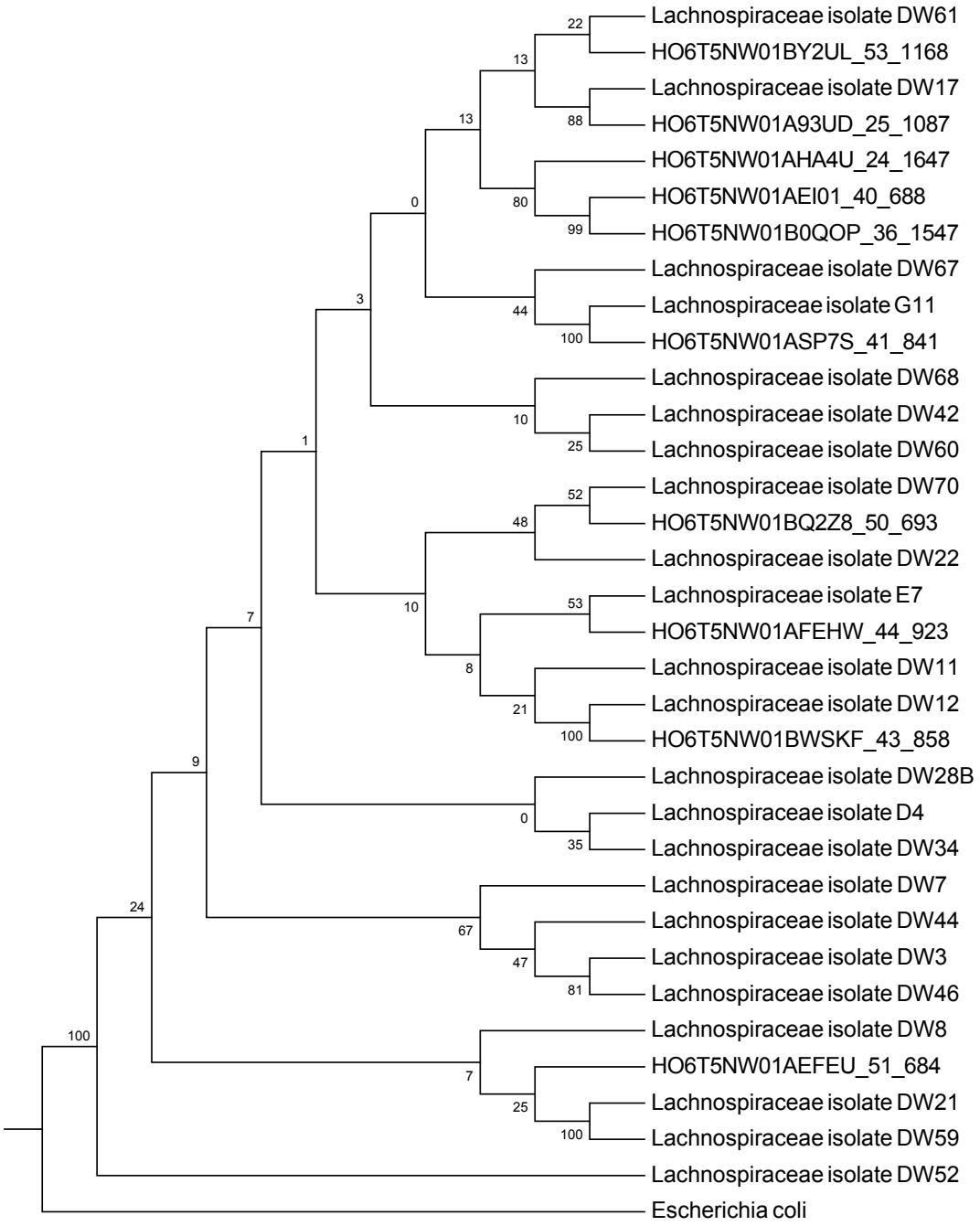


Figure 1. The V3-V5 regions of 16S RNA encoding genes from twenty of our *Lachnospiraceae* isolates, three *Lachnospiraceae* isolates from Reeves, et al.[24] and ten of the most abundant unclassified *Lachnospiraceae* OTUs from Theriot, et al.[35] were used to generate this phylogenetic tree. Bootstrap values indicated are statistical measures corresponding to the probability that members of a given clade are always in that clade.

Direct inhibition

The unperturbed gut microbiota may function in colonization resistance against *C. difficile* by inhibiting its growth. This inhibition may function through a variety of mechanisms: the secretion of microbicides that destroy vegetative *C. difficile*, the competition of space for growth adherence along the gut epithelium, the competition of limiting nutrients or the conversion of host metabolites to compounds that can be inhibitory to *C. difficile* growth[7]. We performed a plate assay, utilizing an overlay technique, to assess each *Lachnospiraceae* isolate's ability to inhibit *C. difficile* growth. If *Lachnospiraceae* isolates secrete anti-*C. difficile* substances, the overlay plate assay will reveal a zone of *C. difficile* clearance around the isolate. With this method, a diluted *C. difficile* culture was allowed to grow over a blot of *Lachnospiraceae* cell culture or supernatant. Direct inhibition was assessed by the presence of a zone of *C. difficile* clearance around the *Lachnospiraceae* blot. A strain of *Enterococcus*, which was shown to directly inhibit *C. difficile* growth on the overlay plate assay, was used as a positive control (unpublished data). Blots of cell culture and of supernatant were used to assess whether cell growth or cell secretions were preventing *C. difficile* growth. All 23 *Lachnospiraceae* isolates, including 3 from Reeves, et al.[24], did not inhibit *C. difficile* growth on the plate assay through either culture blots or supernatant blots.

The same overlay plate technique was performed to assess a group community's ability to inhibit *C. difficile* growth (Figure 2). These communities were those of C57BL/6 mouse cecal content, cecal tissue or feces. Each consisted of *Lachnospiraceae* members and other closely related strains of other families, which were isolated with our 16S rRNA-encoding gene primers specific for *Lachnospiraceae* (Figure 3). For each community, blots of cell culture and supernatant were performed. Similarly to individual *Lachnospiraceae* strains, community cultures of isolates from C57BL/6 mouse cecal content, cecal tissue and feces did not display *C. difficile* inhibition (Table 3).

Since individual isolates and communities of isolates did not exert a direct inhibitory effect on *C. difficile* growth, it is unlikely that there are synergistic effects of our *Lachnospiraceae* isolates in terms of direct inhibition. In other words, the inhibition of *C. difficile* may not necessitate a pathway mediated by multiple *Lachnospiraceae*, or closely related strains. It is unlikely that

multiple *Lachnospiraceae* and closely related microbes cooperatively secrete a *C. difficile* inhibitory product.

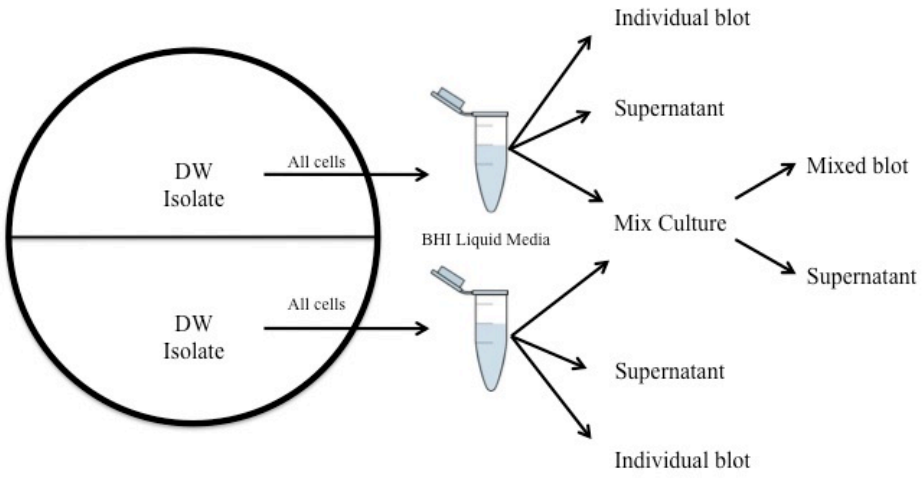


Figure 2. All isolates were used for individual blots in the overlay technique to assess for direct inhibition of *C. difficile*. Individual isolates' cultures and supernatant were tested. Mixed cultures were also created for community testing of direct inhibition of *C. difficile* via cultures or supernatant.

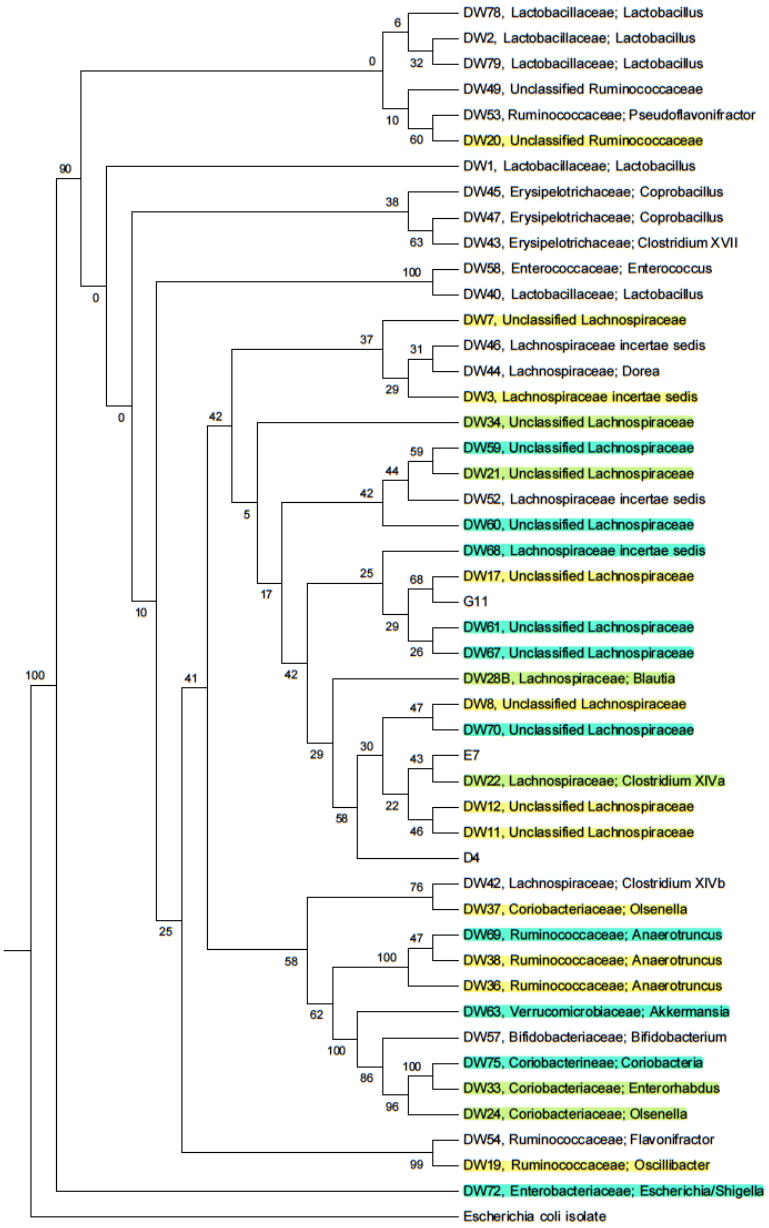


Figure 3. In addition to individual isolates, direct inhibition of community isolates was evaluated. Those isolated from cecal content are highlighted yellow while those from cecal tissue are highlighted green and feces are highlighted blue. The phylogenetic tree demonstrates overlapping diversity of isolates from the three samples sources. Bootstrap values indicated are statistical measures corresponding to the probability that members of a given clade are always in that clade.

	RDP Classification	Direct Inhibition of *C. difficile* by Mixed Culture	Direct Inhibition of *C. difficile* by Mixed Supernatant
Cecal content community members	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Unclassified Lachnospiraceae Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Unclassified Lachnospiraceae Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Unclassified Lachnospiraceae Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Unclassified Lachnospiraceae Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Unclassified Lachnospiraceae Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Unclassified Lachnospiraceae Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; Oscillibacter Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; Unclassified Ruminococcaceae Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; Anaerotruncus Actinobacteria; Coriobacteridae; Coriobacteriale; Coriobacteriaceae; Olsenella Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; Anaerotruncus	-	-
Cecal tissue community members	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Unclassified Lachnospiraceae Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Unclassified Lachnospiraceae Actinobacteria; Coriobacteridae; Coriobacteriales; Coriobacteriaceae; Olsenella Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Blautia Actinobacteria; Coriobacteridae; Coriobacteriales; Coriobacteriaceae; Enterorhabdus Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Unclassified Lachnospiraceae	-	-
Fecal community members	Firmicutes; Clostridia; Clostridiales; Unclassified Clostridiales Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Unclassified Lachnospiraceae Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Unclassified Lachnospiraceae "Verrucomicrobia"; Verrucomicrobiae; Verrucomicrobiales; Verrucomicrobiaceae; Akkermansia Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Unclassified Lachnospiraceae Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Unclassified Lachnospiraceae Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; Anaerotruncus Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Unclassified Lachnospiraceae "Proteobacteria"; Gammaproteobacteria; "Enterobacteriales"; Enterobacteriaceae; Escherichia/Shigella "Actinobacteria"; Actinobacteria; Coriobacteridae; Coriobacteriales; "Coriobacterineae"; Coriobacteriaceae; Enterorhabdus	-	-

Table 3. All strains isolated from different locations of untreated C57BL/6 mice were used to create communities representing cecal content, cecal tissue and feces. These communities were tested for direct inhibition of *C. difficile*. A negative sign (-) indicates a negative result of no direct inhibition of *C. difficile* strain VPI 10463.

Bile acid physiology

Bile salt hydrolase

Indigenous microbes in the GI tract help maintain the balance of bile acids, which are important in *C. difficile* germination and growth[35]. The presence of certain bacteria that can deconjugate germinative primary bile acids (Figure 4) and consequently allow for greater primary to secondary bile acid conversion, can aid in preventing a susceptible state to *C. difficile*. Likewise, the ability to hydrolyze conjugated primary bile acids via bile salt hydrolase is significant in that susceptibility to *C. difficile* colonization is correlated to an increase in primary bile acids and a decrease in secondary bile acids in the gut metabolome[35].

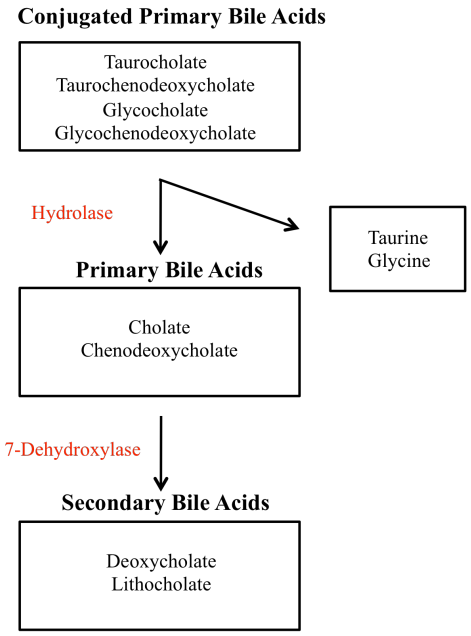
We utilized a bile salt plate assay with conjugated primary bile salts (5% taurocholate and 5% glycocholate) and $CaCl_2$. If bile salt hydrolase activity is present, it will be indicated by the formation of an amino acid-calcium precipitate. Using this procedure, 3 of 17 *Lachnospiraceae* tested had the ability to deconjugate glycocholate and taurocholate into cholate and its respective amino acids[30]. Two of the 17 have potential bile salt hydrolase activity, but further testing is necessary. (Table 4).

7α-dehydrogenase

Once microbes in the GI tract convert conjugated primary bile acids to unconjugated primary bile acids, microbes with dehydrogenase activity convert primary bile acids to secondary bile acids (Figure 4). These secondary bile acids, such as deoxycholic acid (DCA) and lithocholic acid (LCA), are the dominant bile acids present in human feces. While DCA may allow for *C. difficile* spore germination, its effect on the inhibition of *C. difficile* vegetative outgrowth may be more dominant. Additionally, accumulation of secondary bile acids, which affect the composition and the size of the total bile acid pool, has a reciprocal effect on the gut microbiome structure. Disruption of secondary bile acid productivity, by perturbing the gut microbiota, may detrimentally affect human health. A relatively small number of bacteria play a large role in the production of secondary bile acids as only a limited number of intestinal bacteria function in the 7α-dehydroxylation pathway[37]. Thus, it is important to evaluate which members of the gut microbiota can 7α-dehydroxylate primary bile acids.

To assess for 7α-dehydrogenase activity amongst our *Lachnospiraceae* strains, we performed a PCR test with degenerative primers, *baiCD*-F and *baiCD*-R, from Wells, et al.[37] that are specific for conserved regions of the bile acid inducible (*bai*) operon. Although at least 8 proteins are necessary to complete a 7α-dehydrogenation, many of the genes for this complex biochemical pathway are located on the *bai* operon[27,37]. The *baiCD* primers are able to detect low 7α-dehydrogenase activity and are specific to genes only encoding for proteins involved in the dehydrogenase pathway[37].

After PCR screening of all *Lachnospiraceae* isolates, DW 3, DW 7, DW 28 and DW 42 were found to contain *biaCD* genes complementary to the bile acid 7α-dehydroxylase enzyme primers (Figure 5). DNA band sizes were expected to be approximately 1300 base pairs[37]. Positive hits correspond to the expected size. The positive control, *Clostridium scindens* strain VPI 12708, which was used in the *baiCD* primer design, had a strong band at the expected size. Additionally, no band was visible in the negative control.



	Effect on *C. difficile* spore germination	Effect on *C. diffiicile* vegetative outgrowth
Taurocholate	Positive	Unknown
Taurochenodeoxycholate	Unknown	Unknown
Glycocholate	Unknown	Unknown
Glycochenodeoxycholate	Unknown	Unknown
Taurine	Unknown	Unknown
Glycine	Positive	Unknown
Cholate	Positive	Unknown
Chenodeoxycholate	Unknown	Negative
Deoxycholate	Positive	Negative
Lithocholate	Unknown	Unknown

Figure 4. The pathway of conjugated primary bile acids to deconjugated primary bile acids to secondary bile acids produces a variety of effects on *C. difficile* germination and vegetative growth.

Strain	RDP Classification	Bile Salt Hydrolase	7α-dehydrogenase	Isolation Site
DW 3	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Unclassified Lachnospiraceae	-	+	Cecal content
DW 7	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Unclassified Lachnospiraceae	+/-	+	Cecal content
DW 8	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Unclassified Lachnospiraceae	-	-	Cecal content
DW 11	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Unclassified Lachnospiraceae	**NT**	-	Cecal content
DW 12	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Unclassified Lachnospiraceae	**NT**	-	Cecal content
DW 17	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Unclassified Lachnospiraceae	**NT**	-	Cecal content
DW 21	Firmicutes; Clostridia; Clostridiales; Unclassified Clostridiales	-	-	Cecal tissue
DW 22	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Unclassified Lachnospiraceae	+	-	Cecal tissue
DW 28	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Blautia	+/-	+	Cecal tissue
DW 34	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Unclassified Lachnospiraceae	**0**	-	Cecal tissue
DW 42	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Clostridium XlVb	**0**	+	Cecal content
DW 44	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Unclassified Lachnospiraceae	**NT**	-	Cecal content
DW 46	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Unclassified Lachnospiraceae	**NT**	-	Cecal content
DW 52	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Unclassified Lachnospiraceae	**NT**	**NT**	Cecal content
DW 59	Firmicutes; Clostridia; Clostridiales; Unclassified Clostridiales	-	-	Feces
DW 60	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Unclassified Lachnospiraceae	+	-	Feces
DW 61	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Unclassified Lachnospiraceae	**NT**	-	Feces
DW 67	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Unclassified Lachnospiraceae	**0**	-	Feces
DW 68	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Unclassified Lachnospiraceae	-	-	Feces
DW 70	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Unclassified Lachnospiraceae	**0**	-	Feces
D4	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Clostridium XlVa	+	-	Cecal content
E7	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Unclassified Lachnospiraceae	**0**	-	Cecal content
G11	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Unclassified Lachnospiraceae	-	-	Cecal content
DW 40	Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus	+	-	Cecal content

+	Precipitate (resembles positive control)
+/-	Possible precipitate
-	No precipitate
0	Inhibited growth
NT	Not tested

Table 4. We tested 23 *Lachnospiraceae* isolates for 7α-dehydrogenase actitivity using polymerase chain reaction with primers specific for the *baiCD* gene. However, 6 strains of *Lachnospiraceae* were not tested. DW 40, which is 99% similar to *Lactobacilli johnsonii* ZJ626 with which bile salt hydrolase activity has been confirmed[14], was used as a positive control for the bile salt hydrolase assay.

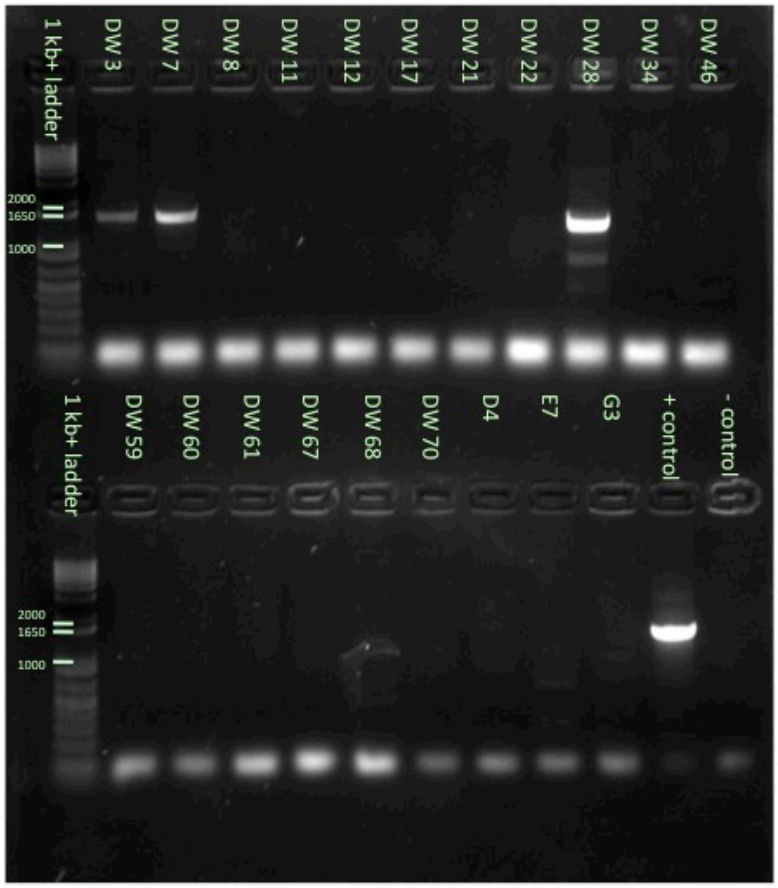


Figure 5. 1% gel electrophoresis shows the presence of genes corresponding to *biaCD*-specific primers in DW 3, DW 7 and DW 28. The positive hit for DW 42 was performed on a separate gel. Positive hits indicate the presence of 7α-dehydrogenase. *Clostridium scindens* VPI 12708 was used as a positive control.

Discussion

The big picture of colonization resistance is an intricate puzzle of numerous factors: the immune response, metabolic environment, microbial competition[7], etc. In order to prevent pathogenesis of organisms such as *C. difficile*, it is necessary to understand how individual microbial species fit into this image. Isolation of key microbes that resist *C. difficile* colonization in the GI tract can lead to the development of treatments that alleviate CDI or that prevent recurrent CDI. However, limited work has been done to examine the organisms responsible for colonization resistance against *C. difficile*. Past work has shown abundance in members of the *Firmicutes* phylum in a *C. difficile*-resistant state. It has also demonstrated the inhibitory effect of one *Lachnospiraceae* isolate in a germfree mouse model against *C. difficile* colonization and toxin production[24]. In order to evaluate the effects of multiple *Lachnospiraceae* in the restoration of colonization resistance, more work is necessary.

To better understand colonization resistance against *C. difficile*, we selectively isolated 20 *Lachnospiraceae* from cecal content, cecal tissue and feces from untreated C57BL/6 mice using plate wash PCR with *Lachnospiraceae*-specific primers. In combination with 3 *Lachnospiraceae* isolated by Reeves, et al.[24], we evaluated 23 *Lachnospiraceae* isolates' abilities to directly inhibit *C. difficile* growth. Furthermore, we analyzed these strains for the presence of bile salt hydrolase activity and for 7α-dehydrogenase activity.

Out of the 23 individual *Lachnospiraceae* isolates tested for direct inhibition against *C. difficile* on the overlay plate assay, none were found to directly inhibit *C. difficile* growth. D4, a *Lachnospiraceae* isolate from Reeves, et al.[24], did not directly inhibit *C. difficile* on the overlay plate assay. Yet, in a germ-free mouse model, it was found to decrease the levels of *C. difficile* colonization and cytotoxin concentration. On the other hand, a strain of *Enterococcus* could directly inhibit *C. difficile* on the overlay plate assay but had little protective effect against *C. difficile* in a germ-free mouse. In addition to individual strains, communities of isolates from cecal tissue, cecal content and feces were evaluated for direct inhibition against *C. difficile* (Table 3). All communities did not directly inhibit *C. difficile* growth. It is possible that the mechanism of colonization resistance is not meditated through the synergistic and cumulative effects of these specific isolates (Figure 3). However, the lack of *C. difficile* inhibition by individual and

communities of strains on the overlay plate assay may be due to discrepancies between the *in vitro* model and *in vivo* occurrences.

Since *in vitro* modeling cannot wholly mimic *in vivo* phenomena, the overlay assay for direct inhibition of *C. difficile* cannot account for a variety of *in vivo* factors. First, even though *Enterococcus* may secrete a substance that is inhibitory to *C. difficile* on the overlay plate assay, this effect may become insignificant *in vivo*. Other metabolites or substances already present in the host's GI tract may deactivate this inhibitory substance before it inhibits *C. difficile* growth. Vice versa, D4 may secrete a substance *in vitro* that does not inhibit *C. difficile*. However, this substance may inhibit *C. difficile* growth in a host through activation by other pre-existing substances of the host's gut. Second, even if *Enterococcus* produces an antimicrobial against *C. difficile*, it may not have an effect on the host's response to *C. difficile*. The kinetics of *Enterococcus* suppression against *C. difficile* growth may not surpass those of the immune system and consequently the onset of CDI symptoms. This may be because *Enterococcus* does not secrete an anti-*C. difficile* substance unless *C. difficile* growth reaches a specific threshold, at which it already has produced TcdA and TcdB. Therefore, TcdA and TcdB disrupt the actin cytoskeleton of epithelial cells, proinflammatory cytokines are released and neutrophil influx lead to inflammation. If *Enterococcus* has secreted a substance that inhibits *C. difficile* growth, it did so too late.

In addition to the inability of an *in vitro* model to take into account the *in vivo* host response, the overlay plate assay for direct inhibition does not factor in nutrient competition or competition for adhesion space along the epithelial lining of the GI tract. In terms of nutrient competition, a better *in vitro* test would utilize a minimal media, rather than a rich media such as BHI that was used in the overlay plate assay. If *Lachnospiraceae* does outcompete *C. difficile* for important and limited nutrients, *C. difficile* growth will be unable to reach stationary phase where its toxin production occurs. This evaluation will be done in future experiments. Lastly, competition between *Lachnospiraceae* and *C. difficile* may be for adhesion space along the epithelial lining of the GI tract. However, as Reeves, et al.[24] suggested, this is unlikely to be the case. Experiments showed that *Lachnospiraceae* D4 restored partial colonization resistance against *C. difficile*, while *Escherichia coli* did not. However, *Escherichia coli* 100-fold higher levels of colonization than *Lachnospiraceae* D4 did in the murine GI tract. As a result, it is thought that space competition

between *Lachnospiraceae* and *C. difficile* in the gut is not an important factor in the role of colonization resistance[24]. Overall, colonization resistance can be mediated by a variety of mechanisms. If direct inhibition is not one, there are others.

Other means of *C. difficile* inhibition by members of the indigenous gut microbiota may have to do with bile salt conversion pathways. Conjugated primary bile acids (termed bile salts when at physiological pH) are produced by the liver, stored in the gall bladder and secreted into the intestinal tract to aid in lipid catabolism during food digestion[26]. These bile salts undergo a series of transformations through the GI tract becoming deconjugated primary bile acids via bile salt hydrolase and finally, secondary bile acids via dehydrogenases[26] (Figure 4). Through this pathway, the gut microbiota plays an essential role in the conversion of bile acids. Likewise, as the balance of bile acid pools in an individual is an important factor in health, the gut microbiota is also responsible for maintenance of a healthy well-being[26].

The effects of bile salt hydrolases and 7α-dehydroxylase on *C. difficile* spore germination and growth, although still in need of further experimental confirmation, vary. Taurocholate, a conjugated primary bile acid, stimulates *C. difficile* spore germination even in its unconjugated bile acid form of cholate. However, after 7α-dehydroxylation of cholate into deoxycholate, two opposite effects are elicited: the stimulation of *C. difficile* spore germination, which is antagonized by its simultaneous inhibition of vegetative cell growth[7]. All in all, despite the multifaceted effects on *C. difficile* a bile salt may have, it is clear that disruption of the gut microbiota with antibiotics creates a disturbed and highly differentiated metabolic environment that allows an individual to become susceptible to *C. difficile* infection. Metabolomic analysis of wild-type mice reveals that completely different microbiome communities of either *Firmicutes* or of *Bacteroidetes* can have identical compositions of bile acids, carbohydrates and fatty acids, which lead to similar *C. difficile*-resistant states[35]. Antibiotic clearing of these bacteria may be responsible for the metabolic shift that allows *C. difficile* to colonize[35]. We sought to identify which of our isolated *Lachnospiraceae* strains are responsible for bile salt hydrolysis and dehydrogenation and that are crucial in maintaining a balanced bile acid pool that aids in *C. difficile* resistance.

Seventeen *Lachnospiraceae* isolates were tested for bile salt hydrolase activity using a bile salt plate assay in which the formation of an amino acid-calcium precipitate indicated bile salt hydrolase activity. With this method, 3 *Lachnospiraceae* isolates were found to cleave bile salts into unconjugated primary bile acids. D4, a *Lachnospiraceae* isolate from murine cecal content[24], was one of the 3. This may indicate that its ability to partially restore colonization resistance against *C. difficile* in a germ-free mouse model is attributable to its ability to hydrolyze, and consequently to reduce the concentration of, conjugated primary bile acids that *C. difficile* needs for germination. We can further test this in our overlay assay, with the addition of conjugated primary bile acids, in which *C. difficile* spore germination is assessed in the presence and absence of *Lachnospiraceae* strains with bile salt hydrolase activity. If the breakdown of bile salts by *Lachnospiraceae* decreases the level of *C. difficile* spore germination, we would observe reduced *C. difficile* growth. Potentially, the combination of all *Lachnospiraceae* isolates with bile salt hydrolase activity may further mediate a higher degree of suppression against *C. difficile* germination and growth. Full colonization resistance may be restored with a complete *Lachnospiraceae* cocktail.

Once primary bile acids are deconjugated, they can be converted to secondary bile acids via the dehydrogenase pathway. We tested all 23 *Lachnospiraceae* strains for the presence of the 7α-dehydrogenase enzyme using a PCR with degenerative primers specific for conserved regions of the bile acid inducible operon. Out of the 4 *Lachnospiraceae* strains found to have 7α-dehydroxylase activity, 3 were isolated from cecal content and 1 was isolated from cecal tissue. The presence of dehydrogenase activity in isolates originating from the large intestine is significant in that vegetative growth and toxin secretion of *C. difficile* cells also occurs here[19]. In colonization resistance, these *Lachnospiraceae* may function to convert primary bile acids to secondary bile acids, such as deoxycholate, that inhibit vegetative *C. difficile* growth. Conversely, the interruption of this function by antibiotic application will decrease the inhibitory effect of secondary bile acids by removing bacteria that are essential in their production.

Although 4 *Lachnospiraceae* isolates were confirmed for 7α-dehydroxylase activity, D4 was not. Additionally, all isolates confirmed to have bile salt hydrolase activity did not have 7α-dehydroxylase acitivity. Vice versa, all isolates confirmed to have 7α-dehydroxylase activitiy did

not have bile salt hydrolase activity. This indicates that the pathway of conjugated primary bile acids to secondary bile acids may not be possible with just 1 *Lachnospiraceae* but through the community effects of multiple *Lachnospiraceae* species. Furthermore, if D4 was able to partially restore colonization resistance without 7α-dehydroxylase, complete colonization resistance may be restored if microbes with dehydroxylase activity are also present.

In addition to direct inhibition and bile salt assessments, additional testing of our *Lachnospiraceae* isolates will further elucidate these organisms' function and role in the gut microbial community's resistance against *C. difficile*. By utilizing a minimal media environment for *Lachnospiraceae* and *Clostridium difficile* growth, we will be able to assess whether certain members of *Lachnospiraceae* are available to outcompete *Clostridium difficile* for a limiting nutrient. Another *in vitro* test is to evaluate whether *Lachnospiraceae* can inhibit the germination of *Clostridium difficile* spores. Additionally, we can model the *in vivo* role of *Lachnospiraceae* in the GI tract using bioreactors, which can be used to replicate the anaerobic environment of a murine GI tract. Consequently, the role of *Lachnospiraceae* in colonization resistance against *C. difficile* can be tested without utilizing an animal model. Ultimately, if the inhibitive potential of our *Lachnospiraceae* isolates is high, a *Lachnospiraceae* "cocktail" will be precolonized in mice, which are later challenged with *C. difficile* inoculation. This will allow us to more accurately measure how *Lachnospiraceae* isolates are involved in *C. difficile* suppression, toxin production and disease severity.

C. difficile is becoming a greater problem in developed countries. The number of patients who suffer and who die from CDI continues to increase. Meanwhile, *C. difficile* treatments are few and limited. Fortunately, previous studies have suggested one *Lachnospiraceae*'s partial protection against *C. difficile* in a germ-free mouse model[24]. While the administration of a single bacterial strain as a probiotic against *C. difficile* mediates limited success, it has been found that defined communities of microbes of relatively little diversity can fully protect against *C. difficile*[6]. Here, a community of *Lachnospiraceae* isolates can provide for a probiotic to protect against *C. difficile*. Our study utilizing 23 novel *Lachnospiraceae* isolates from untreated mice is a promising step towards the development of such a probiotic. These isolates may inhibit *C. difficile* spore germination by hydrolyzing bile salts that *C. difficile* needs. They may also inhibit *C. difficile* growth by maintaining a pool of secondary bile acids. Further testing of these isolates can

definitively reveal the efficacy of a *Lachnospiraceae* cocktail in CDI treatment. Ideally, this *Lachnospiraceae* treatment would not only mitigate CDI symptoms, but also prevent recurrent CDI from occurring, thereby alleviating the nationwide costs of *C. difficile*.

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