2024

Clostridioides difficile: Identification of Rival Organisms & Evaluation of Non-Antibiotic Treatment Implementation

Justin Davis
University of Central Florida, justin.davis@ucf.edu

CLOSTRIDIOIDES DIFFICILE: IDENTIFICATION OF RIVAL ORGANISMS & EVALUATION OF NON-ANTIBIOTIC TREATMENT IMPLEMENTATION

by

Justin Thomas Davis

A thesis submitted in partial fulfillment of the requirements for the Honors Undergraduate Thesis Program in Biomedical Sciences in the College of Medicine Burnett School of Biomedical Sciences and in the Burnett Honors College at the University of Central Florida Orlando, Florida

Spring Term 2024

Thesis Chair: William T. Self, Ph.D.

Abstract

Clostrioides difficile is a common cause of nosocomial (hospital-acquired) infections. Patients receiving antibiotic treatment experience dysbiosis of gut microbiota, and *C. difficile,* normally held in check by the various other organisms, takes this opportunity to propagate. Symptoms of infection generally include diarrhea, colitis, dehydration, and fever. Understanding that *C. difficile* generally only causes illness when it is the dominant bacterium (i.e. when growth is relatively unchecked by other microbes), it is appropriate to investigate potential competitive organisms that may be introduced after antibiotic courses or during active *C. difficile* infection to effectively displace it. Fecal samples from the University of Central Florida Lift fecal collection station were aseptically plated onto modified cycloserine cefoxitin fructose agar (CCFA). Visually remarkable colonies (certain colonies that looked unique in comparison to others) were restreaked on new plates of the same media to verify growth, then transferred to brain heart infusion-supplemented (BHIS) plates for propagation. Colonies were inoculated in glycerol stocks for storage, then grown in BHIS liquid media to prepare for identification. Genomic extraction was performed on each sample, and spectrophotometric quantification and gel electrophoresis were executed to confirm successful extraction. Genomic samples will be sent to an external laboratory for identification via polymerase chain reaction and Sanger sequencing.

We hypothesize that at least one bacterial strain from the fecal collection station will potentially inhibit *C. difficile* infection. Should such an organism be identified, it follows that the efficacy of its application in conventional hospital settings may be examined. Current regulation of fecal microbiota transplants, an effective therapeutic practice, is cumbersome, and changing the classification of fecal transplants may improve timeliness and effectiveness of treatment.

Acknowledgments

I would like to thank my thesis chair, Dr. William T. Self, for his tutelage and mentorship throughout this project. I would also like to acknowledge all the other members of the Self lab who contributed their expertise and friendship; particularly Dominika Dzurny and Michael Johnstone. I would like to thank my thesis committee members, Dr. Robert Borgon, Dr. Alicia Hawthorne, and Dr. Aubrey Jewett, for their advice and input. I appreciate the above named, as well as my friends, coworkers, employers, and professors, for their kindness and support throughout an exciting, hectic, and challenging year. Finally, I would like to thank my parents for their unwavering love, encouragement, and belief in my abilities.

Table of Contents

Figures & Tables

Introduction

1.1 *Clostridioides difficile*

Clostridioides difficile (formerly *Clostridium*) is a Gram-positive, anaerobic, sporogenic bacteria that is a common cause of nosocomial (hospital-acquired) infections and is in fact the leading cause of antibiotic-associated diarrhea, comprising up to 25% of such cases (Barbut & Petit, 2001). *C. difficile* is also responsible for the vast majority of cases of pseudomembranous colitis (Barbut & Petit, 2001). Found in approximately 5% of adults, *C. difficile* spreads through fecal excretion of spores and subsequent oral ingestion (Czepiel et al., 2019).

1.2 Pathogenesis & Current Treatment

Antibiotic treatment generally disrupts the gut microbiota, allowing *C. difficile* to dominate, hence its frequency as a cause of nosocomial infections (Theriot & Young, 2015). Antibiotics effectively rid the gut of most of its existing commensal microbiota, yet the hardy spores of *C. difficile* persevere; they then germinate and become among the first to recolonize the bountiful, competition-free environment (Theriot & Young, 2015). *C. difficile* may also be introduced following antibiotic treatment. The bacterium releases two toxins (A and B) that cause a range of symptoms in the host, most notably colitis and diarrhea (Darkoh et al., 2017). Most current treatments for this illness focus on eliminating *C. difficile* with further antimicrobial (antibiotic) mechanisms that collaterally weaken the host (Darkoh et al., 2017).

1.3 History & Trends

C. difficile was first isolated from the stool of an infant in 1935; the species name stems from its difficulty in culture and isolation (Heinlen & Ballard, 2010). In 1978, it was found to be associated with human disease and responsible for antibiotic-associated diarrhea (Heinlen & Ballard, 2010). In the past decade, *C. difficile* infection (CDI) has increased in both frequency and severity, rendering it one of the most common and troublesome nosocomial microbes (Czepiel et al., 2019). Nearly half a million cases of CDI are reported in the United States each year, and the number of annual CDI-associated deaths ranges between 15,000–30,000, despite a recent uptick in attention and research (Fu et al., 2021).

The >65 age bracket is at significant risk, as symptoms are likely to be stronger and patients are more likely to experience poor outcomes such as death (Czepiel et al., 2019). This risk is compounded by the fact that this age bracket is more likely to spend time in locations that are hotspots for infection, such as hospitals and nursing homes (Schäffler & Breitrück, 2018). However, CDI affects and remains a concern for all age brackets (Czepiel et al., 2019).

1.4 Nutrients

Gut inflammation caused by *C. difficile* triggers the host to produce nutrients to stimulate recovery; *C. difficile* consumes these nutrients and utilizes them to further proliferate (Pruss & Sonnenburg, 2021). Nutrients known to be present during or induced by gut inflammation include the amino acids cysteine, isoleucine, leucine, proline, tryptophan, and valine and carbon sources fructose, glucose, mannitol, sorbitol, and sialic acids (Theriot & Young, 2015). The compound trehalose is regarded to enhance the virulence of *C. difficile* (Collins et al., 2018). Further, recent

research indicates the likelihood of *C. difficile* utilizing uric acid in its growth (Kasahara et al., 2023).

1.5 Objectives & Hypothesis

This project aims to develop a potential new treatment option by identifying at least one competitive organism that consumes the same nutrients as *C. difficile* but is non-toxigenic. If such a microbe could outcompete and essentially starve out *C. difficile* for these nutrients in a replicative environment, it may do the same in a human host. If the competitive microbe produces no toxin, then the host may then experience little to no adverse effect. This is the basis of fecal microbiota transplants; the project aims to identify individual species that may perform well when utilizing this treatment method. We hypothesize that at least one bacterial sample collected from the UCF Lift fecal collection station will show potential to inhibit *C. difficile* infection when incorporated into fecal microbiota transplants.

1.6 Fecal Microbiota Transplants

Nosocomial *Clostridioides difficile* infection is a rising concern in American healthcare, and many alternatives to typical antibiotic courses have been proposed, studied, and implemented. But perhaps existing infection mitigation practices could be better applied: at one urban North Carolina hospital, only 11% of staff followed contact precaution policies in observational studies (Davies & Jolles, 2022). The growing concern of pathogenic bacteria resistant to antimicrobial treatment suggests, according to one source, a clear need for antibiotic stewardship: measures to ensure rational antibiotic treatment based on appropriate drug selection, duration, and route of

administration (Kolář, 2022). Another source indicates that this trend warrants consideration of non-antibiotic solutions to bacterial infections (Opal, 2016).

One such solution involves probiotics, which have been shown to exhibit promising effects in preventing *C. difficile*-associated diarrhea and are safe and tolerable (Al Sharaby et al., 2022). Probiotics can, in fact, be used alongside antibiotics in treatment regimens, and *C. difficile* colitis rates have been shown to be reduced when this treatment method is administered in certain populations (Tegegne & Kebede, 2022). Fecal microbiota transplants are commonly performed to introduce probiotics and have shown to be effective in treating recurrent CDI. In one study, 39 patients with recurrent CDI received a fecal microbiota transplant; 89.7% were successful based on negative toxin testing & culture results 3 months after application (Yeh et al., 2022). Fecal microbiota transplantation is a developing treatment mechanism, and efforts are made to maintain reasonable costs. For instance, application of frozen stool from universal donors reduces expenses to the stool recipient and shortens wait times between development of this treatment plan and the actual infusion (Kim & Gluck, 2019). In addition to treating *C. difficile* infection, fecal microbiota transplants may well be useful in treating other gut-related conditions such as inflammatory bowel disease, obesity, and metabolic syndrome (Gupta et al., 2015).

Despite the shown benefits of fecal transplants, widespread implementation is hindered by a dearth of guidelines and "poorly defined federal regulatory policy" (Vyas, 2015). As of 2015, fecal microbiota transplantation is classified as a drug, preventing physicians from applying this technique in a timely fashion (Vyas, 2015). Reclassification of fecal transplants as human tissue would open the door to more timely and effective transplantation procedures, but it would be important to create restrictive guidelines to ensure patient safety (Vyas, 2015).

Methods

2.1 Research Objectives

The experiment was designed to isolate and identify candidates to outcompete *C. difficile* in a human host, by collection of samples from fecal matter. The first objective was successful isolation, from which six candidates were obtained. The ensuing objectives were species identification and analysis of bacterial yield; the former to determine which candidates were and were not strains of *C. difficile* (toxigenic *C. difficile* transplant would be ill-suited to combat *C. difficile* infection!), the latter to evaluate the candidates' growth yield compared to a *C. difficile* control.

2.2 Modified CCFA Plates

Fecal samples from University of Central Florida Lift fecal collection station were collected in order to attempt to identify a *C. difficile* competitor. These samples were plated onto modified cycloserine cefoxitin fructose agar (CCFA); *C. difficile* is known to grow exceptionally well in this media (George et al., 1979). The utility of CCFA as a selective and differential medium functions to eliminate any microbes that do not survive in the same conditions as *C. difficile*, thus serving as an initial filter to limit growth and ensure that only desired organisms colonize the plates.

C. difficile forms toxins that lead to inflammation and trigger its host to produce nutrients in response; the bacterium subsequently ingests these nutrients and multiplies. The objective of the experiment is to locate a competitive microbe which can be prescribed to symptomatic patients; if a patient is infected with *C. difficile*, then the corresponding nutrients will be present in the

environment at the time of introduction of the prospective microbe. Therefore, plates were made with an excess of certain compounds that *C. difficile* is known or speculated to consume.

The agar contained the standard ingredients of CCFA media in 1 liter of water: proteose peptone (40.0g), fructose (6.0g), disodium phosphate (5.0g), sodium chloride (2.0g), monopotassium phosphate (1.0g), magnesium chloride (0.1g), neutral red (0.03g), cycloserine (250.0mg), cefoxitin (16.0mg), and agar (20.0g). In addition, the agar contained the following nutrients at a concentration of 10 mM: amino acids L-cysteine, L-isoleucine, L-leucine, L-proline, L-tryptophan, and L-valine; carbon sources fructose, trehalose, glucose, mannitol, sorbitol, and sialic acids; and uric acid, a purine product.

2.3 Isolation of Samples

After production of plates with these nutrients, five were set aside for initial application of fecal samples in an anaerobic chamber (~1% H_2, ~5% CO_2, balanced with N_2), within which the following steps were completed. To solicit initial differentiation of bacterial colonies, samples were aseptically plated onto the modified CCFA recipe with nutrients included and grown at 37^o C for 72 hours. Following the initial growth period, the plates were inspected. Eight of the most visually remarkable colonies across each plate were re-streaked onto the modified CCFA and incubated as before to verify growth; seven of these colonies showed substantial growth.

After the subsequent three-day growth period, the remaining seven re-streaked colonies were incubated on BHIS plates for 24 hours at 37°C, encouraging further growth. BHIS is composed of 37 g/L brain heart infusion supplemented with 5 g/L yeast extract and 0.1% L-cysteine. Single colonies were then transferred to 5mL BHIS liquid media (overnights), incubated in the same conditions as the previous step. Six of seven colonies exhibited growth in liquid media;

the seventh was excluded from future steps due to lack of growth. Equal parts of the overnight and 50% glycerol were mixed to form a 25% glycerol stock of each isolate; these six stocks were cataloged and saved for future studies via freezing at −80°C. Samples were sourced from the six stocks in the following identification and analysis procedures.

2.4 Identification of Samples

Step 1. Genomic preparation

Each isolate was streaked out from glycerol stocks onto individual BHIS plates, and incubated for 24 hours in the anaerobic chamber at 37°C. The next day, overnights of each isolate were made by inoculating one colony in separate 5mL BHIS liquid media. These were incubated in the same conditions as the previous step, for subsequent genomic purification adapted from "Gram-Positive Bacteria DNA Purification Protocol" from the GeneJET Genomic DNA Purification Kit by ThermoFisher Scientific. Overnight cultures were added to 1.7mL microcentrifuge tubes by pipetting 1.5mL of culture into an empty tube (*C. difficile* R20291 used as a control).

Samples were centrifuged for 10 minutes at 5,000 × g and the supernatant discarded. Pellets were resuspended with 180µl of lysis buffer, and incubated at 37°C for 30 minutes. 200µl of lysis solution and 20µl of proteinase K were added, then mixed thoroughly by vortexing. Samples were incubated in a water bath for 30 minutes at 56°C, mixed every 10 minutes. 20µl of RNAse A was added to each sample and mixed by vortexing, then incubated for 10 minutes at room temperature. 400µl of 50% ethanol solution was added to each sample and mixed thoroughly.

The lysates were transferred to the GeneJET Genomic DNA Purification Column, inserted in collection tubes, and centrifuged for 1 minute at 6000 × g. Columns were placed into new 2mL

collection tubes, and the old tubes were discarded. 500µl of Wash Buffer I (with ethanol added) was pipetted around the rim of the collection tube, then samples were centrifuged for 3 minutes at 8000 × g and the flow-through was discarded. 500µl of Wash Buffer II was added directly into the tubes and centrifuged for 3 minutes at max speed. Flow-through was discarded, then the tubes were spun for 1 minute at maximum speed to remove all residual solution.

Each column (sample) was transferred directly into a new, sterile 1.5mL microcentrifuge tube, and 50µl of elution buffer was added to each, directly on the center of the membrane. The samples were then incubated at room temperature for 2 minutes, then centrifuged for 1 minute at maximum speed. Eluted samples were recovered from the microcentrifuge tubes and pipetted directly on the membrane of the collection tube, then centrifuged for 1 minute at maximum speed; this step ensures recovery of as much genomic DNA as possible. The samples were stored in their respective microcentrifuge tubes at -20°C until needed, and the purification columns were discarded.

Step 2. Gel electrophoresis

A 1% agarose gel was prepared by mixing 400 mg of agarose in 40 mL of 1x Tris acetate-EDTA (TAE) buffer, then microwaved until the agarose was fully dissolved. The solution was subsequently cooled at room temperature, then 2µl of ethidium bromide was added and mixed in. The gel was poured into a prepared gel cast. As it solidified, samples were prepared for loading: 3µl of genomic sample was mixed with 2µl of loading dye for each target (6 isolates + *C. difficile* R20291 control). Each of the full 5µl mixtures were loaded into their respective well, and the system was ran in 1x TAE buffer for 30 minutes at 100 volts. The gel was subsequently imaged in a Bio-Rad ChemiDoc XRS+ gel imaging system and the resulting image downloaded.

Step 3. DNA quantification via spectrophotometry

Eluted samples were recovered for concentration quantification on a NanoDrop spectrophotometer, measuring absorbance at 260nm. Resulting concentrations were recorded to estimate DNA concentration to ensure samples are within an appropriate range for genome sequencing.

Step 4. Genomic sequencing & identification

After isolation of genomic DNA—confirmed by gel electrophoresis and quantification via spectrophotometry—samples are sequenced after amplification by Polymerase Chain Reaction to determine the genus and species. This step will be completed by an external laboratory, and results are forthcoming at the time of thesis completion.

2.5 Analysis of growth yield

Each of six isolates, along with a *C. difficile* R20291 control, were incubated to compare yields across three replicates. Samples were collected from glycerol stocks and inoculated into 1mL BHIS liquid media tubes, with 1 loopful per tube. Triplicates of each isolate —labeled A, B, and C—were performed, for a total of 21 tubes (6 isolates + *C. difficile* control). Tubes were incubated in the anaerobic chamber at 37°C for 24 hours.

The next day, samples were removed and diluted to a 1:10 sample concentration in BHIS broth; this step was performed to ensure accurate measurement by the spectroscopy machine (Agilent 8453 UV-visible Spectroscopy System). Optical density was measured at 600 nm (OD600) for each tube; two measurements were taken successively for each tube. This step

(measuring optical density) was performed to quantify the turbidity of samples, which corresponds to the amount of bacteria present. Optical density measurements were then multiplied by 10 to reflect the optical density prior to dilution. The process was repeated for three sets of replicates.

An unpaired (independent samples) two-tailed t-test was performed to compare the mean yield for each diluted triplicate against the mean diluted control yield (mean X-A,B,C vs mean Control-A,B,C), with df=4 and significance set at $p<0.05$. The objective is to find specimens that grow well in comparison to *C. difficile*, not necessarily in comparison to each other. Only the first measurement for each tube was included in the calculation; the second measurement was taken to verify accuracy of the first. This test was performed for each set of replicates (i.e. one t-test for each Set 1 sample, separate t-test for Set 2 samples, third t-test for Set 3 samples).

Results

Figure 1. Gel electrophoresis

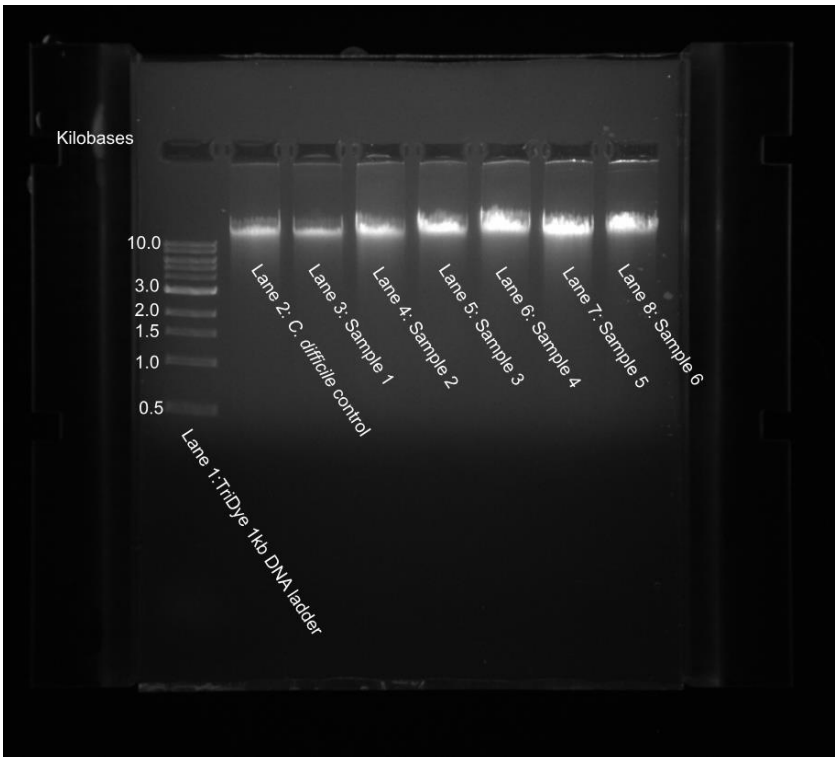

Table 1. Spectrophotometry measurements

Sample	Concentration (ng/µl)
C. difficile R20291 control	85.4
Sample 1	196.3
Sample 2	101.8
Sample 3	119.1
Sample 4	124.2
Sample 5	91.3
Sample 6	123.2

Table 2. Optical density, set 1 (df=4)

Sample	OD 1 (used in calculation)	OD 2 (not used in calculation)
Control A	1.5581	1.5594
Control B	1.6499	1.6459
Control C	2.0700	2.0646
1 A	2.0958	2.0722
1 B	1.9091	1.8912
1 C	2.1702	2.1664
2 A	3.4635	3.4606
2 B	3.0028	3.0001
2 C	3.2407	3.2469
3 A	2.3681	2.3690
3 B	2.3690	2.3678
3 C	2.4246	2.4238
4 A	2.0935	2.1067
4 B	2.2269	X
4 C	2.5268	2.5352
5 A	2.7210	2.7360
5 B	2.8382	2.8297
5 C	2.9411	2.9488
6 A	2.2156	2.2086
6 B	2.4696	2.4694
6 C	2.3867	2.3828

Figure 2. Optical density graph, set 1

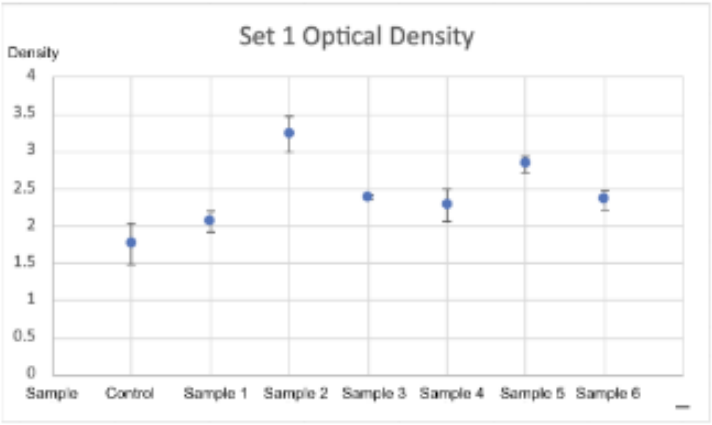

Table 3. Unpaired two-tailed t-test, set 1

	Control	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
Mean	1.7593	2.0584	3.2356	2.3872	2.2824	2.8334	2.3573
St.Dev.	0.2729	0.1345	0.2304	0.0324	0.2219	0.1101	0.1295
t-value		1.7022	7.1592	3.9570	2.5755	6.3211	3.4283
p-value		0.1639	0.0020	0.0167	0.0616	0.0032	0.0266
Significant difference from control?		No	Yes	Yes	No	Yes	Yes

Table 4. Optical density, set 2 (df=4)

Sample	OD 1 (used in calculation)	OD 2 (not used in calculation)
Control A	2.3136	2.3119
Control B	2.3236	2.3338
Control C	2.2027	2.2015
1 A	2.5585	2.5930
1 B	2.4202	2.4279
1 C	2.4313	2.4440
2 A	3.1376	3.1449
2 B	3.0547	3.0627
2 C	3.0302	3.0701
3 A	3.1753	3.1866
3 B	2.8401	3.8456
3 C	3.0300	3.0340
4 A	3.0035	2.9943
4 B	3.1916	3.1929
4 C	2.7662	2.7662
5 A	3.3467	3.3585
5 B	3.2225	3.2215
5 C	3.2473	3.2498
6 A	2.7966	2.8068
6 B	2.9091	2.9126
6 C	2.7833	2.7883

Figure 3. Optical density graph, set 2

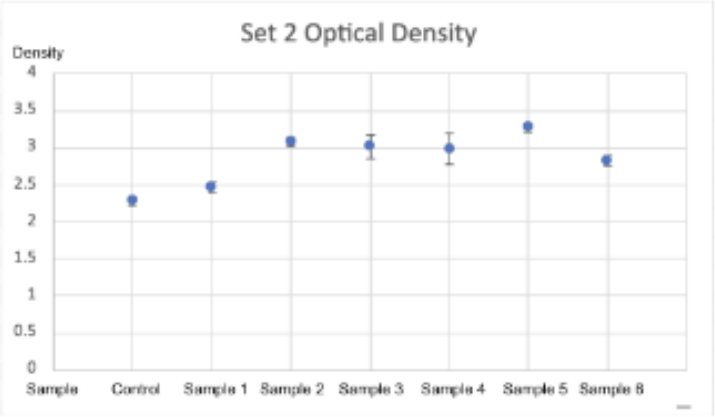

Table 5. Unpaired two-tailed t-test, set 2

	Control	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
Mean	2.2799	2.4700	3.0741	3.0151	2.9871	3.2722	2.8297
St.Dev.	0.0671	0.0768	0.0563	0.1681	0.2312	0.0657	0.0691
t-value		3.2264	15.7065	7.0354	5.4804	18.2960	9.8840
p-value		0.0321	<0.0001	0.0022	0.0054	<0.0001	0.0006
Significant difference from control?		Yes	Yes	Yes	Yes	Yes	Yes

Table 6. Optical density, set 3 (df=4)

Sample	OD 1 (used in calculation)	OD 2 (not used in calculation)
Control A	2.3339	2.3378
Control B	2.6615	2.6637
Control C	2.0550	2.0569
1 A	2.3100	2.3371
1 B	2.2682	2.2831
1 C	2.3489	2.4055
2 A	2.6685	2.6761
2 B	2.8298	2.8395
2 C	3.3242	3.3290
3 A	2.7898	2.8094
3 B	3.3479	3.3459
3 C	3.2871	3.2894
4 A	3.1849	3.1831
4 B	3.1890	3.1857
4 C	2.3794	2.3802
5 A	3.6665	3.6677
5 B	4.0310	4.0356
5 C	4.0189	4.0188
6 A	3.2856	3.2804
6 B	3.2220	3.2256
6 C	2.7412	2.7376

Figure 4. Optical density graph, set 3

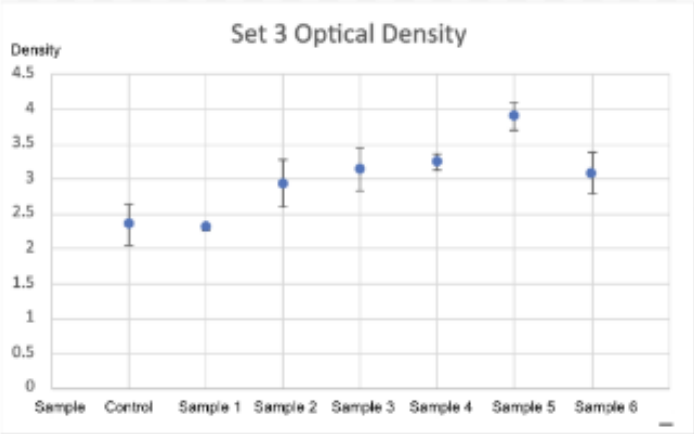

Table 7. Unpaired two-tailed t-test, set 3

	Control	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
Mean	2.3501	2.3090	2.9048	3.1416	3.2511	3.9055	3.0829
St.Dev.	0.3036	0.0404	0.3417	0.3062	0.1111	0.2070	0.2977
t-value		0.2325	2.2386	3.1794	3.8818	7.3313	2.9854
p-value		0.8276	0.0888	0.0336	0.0178	0.0018	0.0405
Significant difference from control?		No	No	Yes	Yes	Yes	Yes

Discussion

Following initial application of collected samples and restreaking of eight visually remarkable colonies, sustained growth was present in six; these six colonies were tested in subsequent experiments. Gel electrophoresis visualized notable quantities of DNA across all six samples and a *C. difficile* control. Electrophoresis served only to verify that DNA was present, pure, and extracted correctly. This outcome was supported by testing in a NanoDrop spectrophotometer. Each of the six experimental samples and control were found to be in an acceptable range to be submitted for sequencing and species identification by an external laboratory; those results are forthcoming at the time of writing.

Experimental samples were compared to controls to determine statistical differences in growth yield. Following 24-hour incubation and optical density measurements, unpaired two-tailed t-tests were performed on each sample versus the control. Statistically significant differences from control were observed for four of six samples in the first set: #2, 3, 5, and 6. In the second set, statistically significant differences from control were observed for all experimental samples, and in the third set, significant differences from control were observed in samples #3, 4, 5, and 6. Of course, some or all of the experimental samples may in fact be strains of *C. difficile;* sequencing results should provide more clarity.

Samples were diluted in BHIS broth because optical density in undiluted tubes was too high to be accurately measured by the spectroscopy machine. In all three sets, each of the experimental samples returned a higher mean yield than their corresponding controls, indicating that the control did not propagate as bountifully as the others despite being incubated in the same conditions. In the first set, the second OD measurement for sample 4B was erroneously skipped; this did not affect the t-test because only initial OD measurements were used to calculate figures.

The study may be repeated without addition of nutrients to CCFA agar; those nutrients are not necessarily mandatory for the overall success of the experiment or even that step, but were included to select for *C. difficile*-like microbes with similar diets. Further, the number of visually remarkable candidates selected for further inspection does not need to be eight; that number was chosen to afford a wide range of potential specimens while maintaining manageability. The experiment could also be adapted to identify experimental samples prior to yield analysis.

Conclusion

Our experiment yielded six tangible samples, all of which showed a sustained capacity for growth. Samples 3, 5, and 6 appear most promising, as they exhibited significantly larger propagation compared to *C. difficile* control in growth yield trials. Future research will include identification of our six collected samples; if any or all are not *C. difficile*, it may be worthwhile to directly test their competitive ability against *C. difficile* in an *in vitro* environment replicative of the human host. This will precede competition testing in animal environments, then finally in human clinical trials. Replicative studies performed in different locations with different initial samples may identify many good competitors, with the ultimate aim of incorporation into non-antibiotic treatment plans.

Fecal microbiota transplants have shown to be successful in clinical studies, but are hindered by a burdensome federal regulation. Reclassification as a human tissue would expand this avenue of treatment, given measures to ensure safety and effectiveness are implemented. This project aims to identify a competitive microbe that may be assimilated into fecal microbiota transplants, providing more specificity to this treatment mechanism. Rather than simply transplanting general stool samples, addition of a known competitive organism to the sample would provide greater assurance that an appropriate microbe will be introduced to the patient.

References

Alrawashdeh, M., Rhee, C., Hsu, H., Wang, R., Horan, K., & Lee, G. M. (2021). Assessment of Federal Value-Based Incentive Programs and In-Hospital *Clostridioides difficile* Infection Rates. *JAMA Network Open*, *4*(10), e2132114. https://doi.org/10.1001/jamanetworkopen.2021.32114

Al Sharaby, A., Abugoukh, T. M., Ahmed, W., Ahmed, S., & Elshaikh, A. O. (2022). Do probiotics prevent clostridium difficile-associated diarrhea? *Cureus*. https://doi.org/10.7759/cureus.27624

Barbut, F., & Petit, J.-C. . (2001). Epidemiology of Clostridium difficile-associated infections. *Clinical Microbiology and Infection*, *7*(8), 405–410. https://doi.org/10.1046/j.1198-743x.2001.00289.x

Blixt, T., Gradel, K. O., Homann, C., Seidelin, J. B., Schønning, K., Lester, A., Houlind, J., Stangerup, M., Gottlieb, M., & Knudsen, J. D. (2017). Asymptomatic Carriers Contribute to Nosocomial Clostridium difficile Infection: A Cohort Study of 4508 Patients. *Gastroenterology*, *152*(5), 1031-1041.e2. https://doi.org/10.1053/j.gastro.2016.12.035

CDC. (2020, March 30). *CDC is always working to protect you from C. diff (deadly diarrhea).* Centers for Disease Control and Prevention. https://www.cdc.gov/cdiff/reducing.html

Collins, J., Robinson, C., Danhof, H., Knetsch, C. W., van Leeuwen, H. C., Lawley, T. D., Auchtung, J. M., & Britton, R. A. (2018). Dietary trehalose enhances virulence of epidemic Clostridium difficile. *Nature*, *553*(7688), 291–294. https://doi.org/10.1038/nature25178

Czepiel, J., Dróżdż, M., Pituch, H., Kuijper, E. J., Perucki, W., Mielimonka, A., Goldman, S., Wultańska, D., Garlicki, A., & Biesiada, G. (2019). Clostridium difficile infection: review. *European Journal of Clinical Microbiology & Infectious Diseases*, *38*(7), 1211–1221. https://doi.org/10.1007/s10096-019-03539-6

Darkoh, C., Deaton, M., & DuPont, H. L. (2017). Nonantimicrobial drug targets for Clostridium difficile infections. *Future Microbiology*, *12*(11), 975–985. https://doi.org/10.2217/fmb-2017-0024

Davies, E., & Jolles, D. (2022). Safe prevention of*clostridium difficile*using infectious disease guidelines at an Urban Hospital in North Carolina. *BMJ Open Quality*, *11*(4). https://doi.org/10.1136/bmjoq-2018-000618

Fletcher, J. R., Pike, C. M., Parsons, R. J., Rivera, A. J., Foley, M. H., McLaren, M. R., Montgomery, S. A., & Theriot, C. M. (2021). Clostridioides difficile exploits toxin-mediated inflammation to alter the host nutritional landscape and exclude competitors from the gut microbiota. *Nature Communications*, *12*(1), 462. https://doi.org/10.1038/s41467-020-20746-4

Fu, Y., Luo, Y., & Grinspan, A. M. (2021). Epidemiology of community-acquired and recurrent Clostridioides difficile infection. *Therapeutic Advances in Gastroenterology*, *14*, 175628482110162. https://doi.org/10.1177/17562848211016248

George, W. L., Sutter, V. L., Citron, D., & Finegold, S. M. (1979). Selective and differential medium for isolation of Clostridium difficile. *Journal of Clinical Microbiology*, *9*(2), 214–219. https://doi.org/10.1128/jcm.9.2.214-219.1979

Goldstein, E. J. C., Johnson, S., Maziade, P.-J. ., McFarland, L. V., Trick, W., Dresser, L., Millette, M., Mazloum, H., & Low, D. E. (2015). Pathway to Prevention of Nosocomial Clostridium difficile Infection. *Clinical Infectious Diseases*, *60*(suppl 2), S148–S158. https://doi.org/10.1093/cid/civ142

Gupta, S., Allen-Vercoe, E., & Petrof, E. O. (2015). Fecal microbiota transplantation: In perspective. *Therapeutic Advances in Gastroenterology*, *9*(2), 229–239. https://doi.org/10.1177/1756283x15607414

Gursoy, S., Guven, K., Arikan, T., Yurci, A., Torun, E., Baskol, M., Ozbakir, O., & Yucesoy, M. (2007). Clostridium difficile infection frequency in patients with nosocomial infections or using antibiotics. *Hepato-Gastroenterology*, *54*(78), 1720–1724. https://pubmed.ncbi.nlm.nih.gov/18019703/

Heinlen, L., & Ballard, J. D. (2010). Clostridium difficile infection. *The American Journal of the Medical Sciences*, *340*(3), 247–252. https://doi.org/10.1097/maj.0b013e3181e939d8

Hsu, H. E., Wang, R., Broadwell, C., Horan, K., Jin, R., Rhee, C., & Lee, G. M. (2020). Association Between Federal Value-Based Incentive Programs and Health Care–Associated Infection Rates in Safety-Net and Non–Safety-Net Hospitals. *JAMA Network Open*, *3*(7), e209700. https://doi.org/10.1001/jamanetworkopen.2020.9700

Kasahara, K., Kerby, R. L., Zhang, Q., Pradhan, M., Mehrabian, M., Lusis, A. J., Göran Bergström, Fredrik Bäckhed, & Rey, F. E. (2023). Gut bacterial metabolism contributes to host global purine homeostasis. *Cell Host & Microbe*, *31*(6), 1038-1053.e10. https://doi.org/10.1016/j.chom.2023.05.011

Kim, K. O., & Gluck, M. (2019). Fecal microbiota transplantation: An update on clinical practice. *Clinical Endoscopy*, *52*(2), 137–143. https://doi.org/10.5946/ce.2019.009

Kolář, M. (2022). Bacterial infections, antimicrobial resistance and antibiotic therapy. *Life*, *12*(4), 468. https://doi.org/10.3390/life12040468

Lawson, P. A., Citron, D. M., Tyrrell, K. L., & Finegold, S. M. (2016). Reclassification of Clostridium difficile as Clostridioides difficile (Hall and O'Toole 1935) Prévot 1938. *Anaerobe*, *40*, 95–99. https://doi.org/10.1016/j.anaerobe.2016.06.008

McFarland, L. V., Mulligan, M. E., Kwok, R. Y. Y., & Stamm, W. E. (1989). Nosocomial Acquisition of Clostridium difficile Infection. *New England Journal of Medicine*, *320*(4), 204–210. https://doi.org/10.1056/nejm198901263200402

Opal, S. M. (2016). Non-antibiotic treatments for bacterial diseases in an era of progressive antibiotic resistance. *Critical Care*, *20*(1). https://doi.org/10.1186/s13054-016-1549-1

Policy (OIDP), O. of I. D. and H. (2021, September 2). *HAI National Action Plan*. HHS.gov. https://www.hhs.gov/oidp/topics/health-care-associated-infections/hai-action-plan/index.html

Pruss, K. M., & Sonnenburg, J. L. (2021). C. difficile exploits a host metabolite produced during toxin-mediated disease. *Nature*, *593*(7858), 261–265. https://doi.org/10.1038/s41586-021-03502-6

Reagan, J., Herzig, C. T. A., Pogorzelska-Maziarz, M., Dick, A. W., Stone, P. W., & Divya Srinath, J. (2015). State Law Mandates for Reporting of Healthcare-Associated Clostridium difficile Infections in Hospitals. *Infection Control and Hospital Epidemiology*, *36*(3), 350–352. https://doi.org/10.1017/ice.2014.61

Schäffler, H., & Breitrück, A. (2018). Clostridium difficile – From Colonization to Infection. *Frontiers in Microbiology*, *9*(646). https://doi.org/10.3389/fmicb.2018.00646

Solanky, D., Juang, D. K., Johns, S. T., Drobish, I. C., Mehta, S. R., & Kumaraswamy, M. (2020). Using diagnostic stewardship to reduce rates, healthcare expenditures and accurately identify cases of hospital-onset *Clostridioides difficile* infection. *Infection Control & Hospital Epidemiology*, *42*(1), 51–56. https://doi.org/10.1017/ice.2020.375

Tegegne, B. A., & Kebede, B. (2022). Probiotics, their prophylactic and therapeutic applications in human health development: A review of the literature. *Heliyon*, *8*(6). https://doi.org/10.1016/j.heliyon.2022.e09725

Theriot, C. M., & Young, V. B. (2015). Interactions Between the Gastrointestinal Microbiome and Clostridium difficile. *Annual Review of Microbiology*, *69*(1), 445–461. https://doi.org/10.1146/annurev-micro-091014-104115

Vaughn, V. M., Saint, S., Greene, M. T., Ratz, D., Fowler, K. E., Patel, P. K., & Krein, S. L. (2020). Trends in Health Care–Associated Infection Prevention Practices in US Veterans Affairs Hospitals From 2005 to 2017. *JAMA Network Open*, *3*(2), e1920464. https://doi.org/10.1001/jamanetworkopen.2019.20464

Villafuerte-Gálvez, J., Pollock, N. R., Alonso, C. D., Chen, X., Xu, H., Wang, L., White, N., Banz, A., Miller, M. A., Daugherty, K., Gonzalez-Luna, A. J., Barrett, C., Sprague, R., Garey, K. W., & Kelly, C. P. (2022). Stool Interleukin-1β Differentiates *Clostridioides difficile* Infection (CDI) From Asymptomatic Carriage and Non-CDI Diarrhea. *Clinical Infectious Diseases*, *76*(3), e1467–e1475. https://doi.org/10.1093/cid/ciac624

Vyas, D. (2015). Fecal transplant policy and legislation. *World Journal of Gastroenterology*, *21*(1), 6. https://doi.org/10.3748/wjg.v21.i1.6

Wilson, K. H., & Perini, F. (1988). Role of competition for nutrients in suppression of Clostridium difficile by the colonic microflora. *Infection and Immunity*, *56*(10), 2610–2614. https://doi.org/10.1128/iai.56.10.2610-2614.1988

Yeh, Y.-M., Cheng, H.-T., Le, P.-H., Chen, C.-C., Kuo, C.-J., Chen, C.-L., Chiu, C.-T., & Chiu, C.-H. (2022). Implementation of fecal microbiota transplantation in a medical center for recurrent or refractory clostridioides difficile infection and report of preliminary outcome. *Biomedical Journal*, *45*(3), 504–511. https://doi.org/10.1016/j.bj.2021.06.001

Zhu, D., Sorg, J. A., & Sun, X. (2018). Clostridioides difficile Biology: Sporulation, Germination, and Corresponding Therapies for C. difficile Infection. *Frontiers in Cellular and Infection Microbiology*, *8*(29). https://doi.org/10.3389/fcimb.2018.00029