A Potential Solution to a Poopy Problem: Bile Salt Analogs as Prophylactics Against Clostridium Difficile Infection

Jacqueline Renee Phan

University of Nevada, Las Vegas, jacquelinerphan@gmail.com

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A POTENTIAL SOLUTION TO A POOPY PROBLEM: BILE SALT ANALOGS AS PROPHYLACTICS AGAINST CLOSTRIDIUM DIFFICILE INFECTION (CDI)

by

Jacqueline Renee Phan

Bachelor of Science in Biological Sciences
University of Nevada, Las Vegas
2012

Bachelor of Science in Biochemistry
University of Nevada, Las Vegas
2013

A thesis submitted in partial fulfillment of the requirements for the

Master of Science – Biochemistry

Department of Chemistry & Biochemistry
College of Sciences
The Graduate College

University of Nevada, Las Vegas
December 2017
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Thesis Approval
The Graduate College
The University of Nevada, Las Vegas
September 7, 2017

This thesis prepared by

Jacqueline Renee Phan

entitled

A Potential Solution to a Poopy Problem: Bile Salt Analogs as Prophylactics against Clostridium difficile Infection (CDI)

is approved in partial fulfillment of the requirements for the degree of

Master of Science – Biochemistry
Department of Chemistry & Biochemistry

Ernesto Abel-Santos, Ph.D.
Examination Committee Chair

Kathryn Hausbeck Korgan, Ph.D.
Graduate College Interim Dean

Ronald Gary, Ph.D.
Examination Committee Member

Bryan Spangelo, Ph.D.
Examination Committee Member

Jefferson Kinney, Ph.D.
Graduate College Faculty Representative
ABSTRACT

A Potential Solution to a Poopy Problem: Bile Salt Analogs as Prophylactics Against Clostridium Difficile Infection (CDI)

by

Jacquelin Renee Phan

Dr. Ernesto Abel-Santos, Examination Committee Chair
Professor of Biochemistry
University of Nevada, Las Vegas

Clostridium difficile infection (CDI) is a major cause of antibiotic-associated diarrhea. In 2011, over 500,000 patients were diagnosed with CDI in the United States and over 29,000 people died of CDI-related complications. With an average of $35,000 to treat a single case of inpatient CDI, cost burden to the healthcare system can reach up to $3.2 billion annually. As both hospital- and community-acquired CDI incidences rise due to the emergence of hypervirulent strains and CDI reoccurrences of up to 25%, standard treatments are rendered less effective and new methods of prevention are critical.

CDI is caused by bacteria called Clostridium difficile. A key characteristic of Clostridium difficile is its ability to form tough and dormant structures called spores. The spores’ dormant nature allows them to survive in the gastrointestinal tract of susceptible patients without showing any signs of infection. When the spores are under stress, they can germinate into toxin-producing cells that cause symptomatic infection.
Clostridium difficile spore germination is promoted by the bile salt taurocholate with the amino acid glycine. Another naturally-occurring bile salt called chenodeoxycholate (CDCA) can compete with taurocholate to inhibit spore germination. These bile salts are regulated by the indigenous gut microbiota. However, for patients who are immunocompromised or who have recently taken antibiotics, the composition of natural intestinal microflora can become altered, making bile salt regulation much less efficient, thus allowing spore germination to occur.

Previously, CamSA, a synthetic bile salt analog of taurocholate, was found to be a more potent germination inhibitor than CDCA when tested against epidemic type X strain 630. Currently, a new analog called 07C revealed to be a stronger germination inhibitor than CamSA in strain 630 as well as in various other strains. Plated germination inhibition assays showed that 07C inhibited spore germination in several strains of C. difficile using less than 50 μM of compound. Furthermore, mice challenged with each of the C. difficile strains had significantly reduced CDI symptoms or were completely protected from CDI symptoms when given three doses of 50 mg/kg 07C. From these explorations, bile salt analogs have the potential to serve as CDI prophylactic treatments in antibiotic-treated patients.
ACKNOWLEDGEMENTS

A famous African proverb once said, “It takes a village to raise a child.”

Walking into graduate school, I felt completely overwhelmed and unsure about how to take my first steps. However, over the past few years, I have received tremendous guidance and support from so many wonderful individuals who have helped me grow along the road to completing my graduate studies.

First and foremost, I would like to thank Dr. Ernesto Abel-Santos for his ongoing patience and guidance throughout my graduate career. Through his mentorship, I have built the confidence to tackle many new and challenging obstacles that I never thought were possible for me to accomplish. Most of all, I would like to thank him for taking a chance with me and helping me realize my true potential.

I am very grateful to my committee members who have given me invaluable time, insight, and advice. Dr. Ronald Gary opened his doors to me whenever I had any questions or concerns, Dr. Bryan Spangelo offered me many tips on how to make my graduate studies flow smoothly, and Dr. Jefferson Kinney provided my research with a fresh, yet seasoned, perspective.

Thank you to the UNLV Graduate College for providing me with many opportunities to showcase my research to the rest of the community. I appreciate the support and the time spent in putting events together for the graduate students.
The UNLV Chemistry & Biochemistry Department faculty and staff has been an integral part of my success as a graduate student. Big thanks to Deborah Masters, Mark Miyamoto, and Bianca Rideout who have worked tremendously hard to make graduates students like myself feel comfortable and cared for. Thanks to Dr. David Hatchett and Dr. Spencer Steinberg for their leadership in the department. I am also appreciative of Dr. Kathleen Robins for her warm smile and advice that always make me feel welcome. I would like to extend special appreciation to Keala Kiko for always giving me encouragement and for his diligence with gearing me on the right track to finishing my degree.

I would like to thank Dr. Amber Howerton who I had the pleasure of assisting during her post-doctoral research. I am very lucky to have been paired with her during my first experiences with lab work. It was through Amber’s leadership and mentorship that I realize my unexpected love for research. I want to thank her for taking a leap of faith and passing this important research on to me. I continue to aspire to reach her level of research etiquette and skillfulness.

I also thank Dr. Israel Alvarado for his mentorship and friendship. Through our shared experiences with balancing graduate school and parent life, I felt like I had someone who could relate to my struggles. Israel is kind to everyone in the lab and was a great source of knowledge and encouragement whenever I felt unsure of myself.
Thank you to my fellow Abel-Santos Lab graduate students for their comradery and support. I thank Chandler Hassan for being my study buddy through our didactic classes together and for having my back throughout graduate school. Our funny little conversations helped me get through a long lab work day. I also thank Natiera Magnuson for her thoughtful reminders about lab safety and etiquette and for thinking of your fellow lab members during difficult times. I would like to extend my deepest gratitude to Christopher Yip to whom I would not have been able to complete my graduate studies without. From day one, I bombarded him with a plethora of silly questions and I still continue to till this day. I don’t know how he puts up with me, but nevertheless, I would like to thank him for not only dealing with me, but for saving me so many times by helping me troubleshoot all the little problems associated with the very fickle C. diff, for babysitting my anaerobic chamber when I’m away, for grabbing my mail and gas tanks, for his suggestions on the best coffee shops to visit during lunch breaks, and for his invaluable advice and knowledge. I am forever indebted to him.

Thanks to all the members of “Team Mouse”, my undergraduate assistants: Minh Chau Truong, Connie Ngo, Julian Phan, and Dung “Jenny” Do. I honestly would not be able to finish this degree without them all unselfishly taking time out of their lives to help me with my research. I want to thank them for being there when I call at random times throughout the day to help me check on the mice, make media, or trial a new experiment. The betterment of scientific
research and the scientific community is dependent on those like them and I wish them all the very best on their future endeavors.

I would like to express my gratitude to all the members of the Abel-Santos Lab, both past and present, and to all my fellow graduate students at UNLV that I’ve had the pleasure of working with during my graduate career. It is through the small encounters that I had with each and every one of them that helped me brainstorm and come up with new and exciting ideas. I appreciate their continued support.

Thank you to my parents Jacques (Ba or “Dodo”) and Jenny (Me or “Momo”) for raising me to be the person I am today. I am eternally grateful for the life they have given me. Although they don’t quite understand what I do and why I do it, I would like to thank them for loving me and accepting me unconditionally. Thank you to my sisters Joy and Julian for believing in me and for always having my back. It is because I have two intelligent and hardworking little sisters that I push myself through my triumphs and mistakes to continue to be a good example for them both.

To my “bees”, Allen: there are no words to truly say how thankful I am to have you on this journey with me. Thank you so much for your undying support and love. No one understands my struggles the way you do. Thank you for always allowing me to pursue whatever crazy dream I have and always putting yours on the backburner so that I could try mine first. Thank you for accompanying me to UNLV for events and for staying up until 2 A.M. on your
days off or keeping yourself awake after you get off work at 6 A.M. so that I have company while finishing writing papers or practicing my talks over and over again. Thank you for rearranging your work schedule to work graveyard shifts so that you have time to help me watch the kids during the daytime so I can get work done in the lab. Thank you for reading, proofreading, and giving me advice on my writing drafts even when they don’t really make sense to you. Thank you for trying to learn foreign concepts so that you can better advise me on my work. Thank you for tolerating me when I change a single word in a talk or paper and make you hear it or read it all over again. You have done so much more for me during my graduate studies that it would take the entire length of this thesis for me to write everything down. <3 you.

Thank you to my precious little dudes: Jack and Aiden. You guys have made graduate school challenging, but so worth it. Thanks for all the amazing hugs and kisses I get when I come home. Thank you for sitting in front of me while I go over my talks just so I don’t feel like I’m talking to a wall and for playing on your iPad next to me so I’m not lonely when I’m writing. You two are so sweet and supportive, and are my motivation to do my best every day.

Last, but not least, thanks be to God. “I can do all things through Him who strengthens me.” – Philippians 4:13
DEDICATION

This thesis is dedicated to my guardian angel in heaven, Bà Ngoạị (maternal grandmother) Maria Phạm Thị Lụ, who passed away during my graduate studies on August 11th, 2015. Missing you every day...
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>B.</td>
<td>Bacillus</td>
</tr>
<tr>
<td>BHI</td>
<td>Brain heart infusion</td>
</tr>
<tr>
<td>BHIS</td>
<td>Brain heart infusion supplemented [with yeast]</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BW</td>
<td>Body weight</td>
</tr>
<tr>
<td>C.</td>
<td>Clostridium</td>
</tr>
<tr>
<td>C-terminus</td>
<td>Carboxyl terminus</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>CCNA</td>
<td>Cell cytotoxicity neutralization assay</td>
</tr>
<tr>
<td>CDAD</td>
<td>Clostridium difficile associated diarrhea</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td>CDCA</td>
<td>Chenodeoxycholate</td>
</tr>
<tr>
<td>CDI</td>
<td>Clostridium difficile infection</td>
</tr>
<tr>
<td>CDT</td>
<td>Clostridium difficile transferase</td>
</tr>
<tr>
<td>CFU(s)</td>
<td>Colony forming unit(s)</td>
</tr>
<tr>
<td>CO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Carbon Dioxide</td>
</tr>
<tr>
<td>CPD</td>
<td>Cysteine protease domain</td>
</tr>
<tr>
<td>CROP</td>
<td>Combined repetitive oligopeptide repeat</td>
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<tr>
<td>CSPG4</td>
<td>Chondroitin sulfate proteoglycan 4</td>
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<td>D(#)</td>
<td>Day number</td>
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<tr>
<td>DI</td>
<td>Deionized</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>DNA</td>
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<td>DPA</td>
<td>Dipicolinic acid</td>
</tr>
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<td>Dr.</td>
<td>Doctor</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EtBr</td>
<td>Ethidium Bromide</td>
</tr>
<tr>
<td>FMT</td>
<td>Fecal microbiota transplantation</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
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<tr>
<td>Ger</td>
<td>Germination</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
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<td>Glycoprotein 96</td>
</tr>
<tr>
<td>GTD</td>
<td>Glucosyltransferase</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
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<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Hydrogen gas</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>Water</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>IACUC</td>
<td>Institutional Animal Care and Use Committee</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>sec</td>
<td>Seconds</td>
</tr>
<tr>
<td>SMC</td>
<td>C. difficile sporulation media</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate-EDTA</td>
</tr>
<tr>
<td>TC</td>
<td>Taurocholate</td>
</tr>
<tr>
<td>TcdA</td>
<td>Clostridium difficile toxin A</td>
</tr>
<tr>
<td>TcdB</td>
<td>Clostridium difficile toxin B</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>UDP</td>
<td>Uridine diphosphate</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>WBC</td>
<td>White blood cell</td>
</tr>
<tr>
<td>% w/v</td>
<td>Percent weight of solution in a total volume</td>
</tr>
<tr>
<td>°C</td>
<td>Celsius degree</td>
</tr>
<tr>
<td>μL</td>
<td>Microliter</td>
</tr>
<tr>
<td>μM</td>
<td>Micromolar</td>
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<tr>
<td>× g</td>
<td>Times gravity</td>
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CHAPTER 1

INTRODUCTION

1.1 Background and Significance

*Clostridium difficile* is a pathogenic bacterium linked with *Clostridium difficile* antibiotic-associated diarrhea. Collectively, disease caused by this bacterium is recognized as *Clostridium difficile* infection (CDI). The discovery of *C. difficile* was preceded by the characterization of pseudomembranous colitis (PMC) by John Miller Turpin (J.M.T.) Finney in 1893.¹ PMC is an inflammatory condition in which exudative plaques form on the intestinal mucosa causing necrotizing disease. As a surgeon at Johns Hopkins Hospital, Finney discovered PMC in an autopsy of a postoperative patient who had undergone a gastroenterostomy for an ulcerated pylorus.¹,² Although the patient began to recover ten days post-surgery, the patient soon developed bloody diarrhea and succumbed five days later.¹ Over the next 50 years, other similar cases were reported involving postoperative PMC.²-⁴ By the 1950s, several reports of diarrhea and colitis were associated with prior exposure to antibiotics.⁵-⁸

Meanwhile, in 1935, while investigating the colonization of bacteria in the gastrointestinal (GI) tracts of human neonates, Ivan C. Hall and Elizabeth O'Toole isolated bacteria they named “*Bacillus difficilis*” from the stools of the healthy newborn infants.⁹ The name “*difficilis*” arose from the bacterium’s initial difficulty to isolate and culture from human feces. While working with “*Bacillus difficilis*”, Hall and O'Toole found that the organism was able to produce toxin
that was lethal to guinea pigs.\textsuperscript{9} Interestingly, the infants of which the organism was discovered from were found to be asymptomatic carriers of the bacterium.\textsuperscript{9} Thus, the organism was deemed as commensal. A 2011 comprehensive review by Kachrimanidou and Malisiovas reported that approximately 5\% of healthy adults and 50\% of newborn infants are asymptomatic carriers of \textit{C. difficile}.\textsuperscript{10}

It was not until the 1970s, when the antibiotic clindamycin was first introduced, that “\textit{Bacillus difficultis}”, now renamed to \textit{Clostridium difficile}, was found to be responsible for antibiotic-associated diarrhea and a primary cause of PMC.\textsuperscript{11-14} Moreover, the toxins produced by \textit{C. difficile} were implicated as the causative agents of PMC.\textsuperscript{15-18} By 1996, United States hospital discharges from \textit{Clostridium difficile} antibiotic-associated diarrhea were 31 cases per 100,000 population.\textsuperscript{19,20} This proportion doubled to 61 cases per 100,000 population by 2003.\textsuperscript{19,20}

In 2011, the Centers for Disease Control (CDC) reported that over 500,000 people were diagnosed with CDI in the United States.\textsuperscript{21,22} In that same year, approximately 29,000 people died of CDI-related complications within 30 days of initial diagnosis.\textsuperscript{22} With an average of $35,000 to treat a single inpatient case, cost burden to the United States healthcare system can reach up to $3.2 billion annually.\textsuperscript{22-26} Some studies have even traced CDI as the cause of $4.8 billion in excess cost to United States acute-care facilities.\textsuperscript{27} A 2011 study by Miller et al. suggests that CDI has even surpassed the infamous methicillin-resistant \textit{Staphylococcus aureus} (MRSA) as the most common cause of hospital
associated infections (HAIs) in community hospitals in the southeastern United States. 

1.2 Biology of *Clostridium difficile*

1.2.1 Taxonomy

*Clostridium difficile* is a member the Domain *Bacteria* and Kingdom *Eubacteria* (Figure 1.1). It belongs to the Phylum *Firmicutes*. Like most of the bacteria in Phylum *Firmicutes*, *C. difficile* is Gram-positive, which means that it contains a thick peptidoglycan cell wall that can readily absorb the primary stain crystal violet. Once the stain becomes fixed to the cells upon application of the mordant iodine, the *C. difficile* rods can be viewed as purple under light microscopy (Figure 1.2). By contrast, Gram-negative bacteria, such as *Escherichia coli*, possess a thin peptidoglycan layer sandwiched between an inner cytoplasmic cell membrane and an outer membrane composed of lipopolysaccharides. This causes Gram-negative organisms to not retain the primary stain crystal violet after decolorization with alcohol, but readily uptake the secondary stain safranin. Therefore, they appear pink under light microscopy (Figure 1.2). This staining technique was devised by Danish bacteriologist Hans Christian Gram in 1884 as a means of separating bacteria into two distinct groups based on their cell wall content (Figure 1.3). Phylum *Firmicutes* bacteria are also known for their low percentage of guanine-cytosine (GC)
nitrogenous base content in their genetic information (DNA or RNA). The C. difficile genome has been reported to have a 29.06% GC content.\textsuperscript{\textcopyright 33}

**Figure 1.1.** Classification of Clostridium difficile.

**Figure 1.2.** Gram stain of Gram-positive C. difficile (purple) and Gram-negative E. coli (pink).
Phylum *Firmicutes* consists of two major classes of rod-shaped bacteria: *Bacilli* and *Clostridia* (Figure 1.1). While *Bacilli* can be obligate or facultative aerobes, *Clostridia* are obligate anaerobes. This means that they do not tolerate the presence of oxygen, and thus use nitrate as their final electron acceptor in cellular respiration to produce molecular energy in the form of the adenosine triphosphate (ATP). Therefore, the anaerobic nature of the
mammalian GI tract coupled with its high nutrient availability allows C. difficile the ability to thrive within it.\textsuperscript{37}

C. difficile is further classified into Order Clostridiales and Family Clostridiaceae (Figure 1.1). As with other bacteria in Family Clostridiaceae, C. difficile is able to form structures called endospores.\textsuperscript{30,35} Endospores are very often referred to as “spores” although they are not true spores in the sense that they are not reproductive offspring (such as with plants or fungi), but rather are non-reproductive seed-like entities. Endospores are tough and dormant structures that are formed when the vegetative cell is exposed to harsh or stressful conditions like nutrient deprivation. These spores are highly resistant to various stressors such as high temperatures, ultraviolet irradiation, and a variety of antimicrobial treatments.\textsuperscript{38-40} Their dormant nature also allows them to survive on surfaces such as hospital counters and in the GI tracts of susceptible patients.\textsuperscript{41}

Finally, the genus Clostridium contains about 200 species that consist of non-symbiotic bacteria, commensal bacteria, as well as notorious pathogenic bacteria such as C. botulinum (responsible for botulism), C. perfringens (responsible for gas gangrene), C. tetani (responsible to tetanus), and C. difficile (responsible for CDI) (Figure 1.1).\textsuperscript{34}

C. difficile strains are highly motile and move via peritrichous flagella, which are flagellar extensions that are spread throughout the surface of the bacterium.\textsuperscript{42} However, Baban et al. has shown that C. difficile strain R20291
exhibits monotrichous flagella (a single flagellum on one end of the bacterium). The study suggests that monotrichous flagella assists the strain R20291 bacteria not only in motility, but also in adherence and colonization to the intestinal epithelium upon infection.

### 1.2.2 Sporulation

The production of endospores is a key characteristic of *C. difficile*. The process by which endospores are formed is called sporulation. Sporulation is triggered by stimuli from both the external and internal environment. External cues include quorum sensing, nutrient starvation, and desiccation. Following external stimulus, initiation proteins that comprise of sensory histidine kinases phosphorylate regulatory proteins including the master transcriptional regulator protein Spo0A, which is highly conserved among members of the *Bacillus* and *Clostridium* genera. Spo0A has been found to be an important factor in the transmissivity of CDI and play a significant role in maintenance of persistent infection. The recruitment of initiation and regulatory proteins commence a cascade of sigma (σ) factors and additional sporulation proteins that signal the formation of the many layers of the *C. difficile* endospore.

The overall structure of the *C. difficile* spore is similar that of other spore-forming bacteria in genera *Bacillus* and *Clostridium* (Figure 1.4). However, *C. difficile* lacks some sporulation orthologs that are commonly conserved in other species of those genera. This is particularly apparent in the outer layers of the C.
*C. difficile* spore. The outermost layer of the spore is the exosporium, which contains hair-like proteinaceous projections that help attach the spore to host cells. The exosporium is also the outermost layer in *B. anthracis* and *B. cereus*. Interestingly, *C. difficile* spores that possessed defective or missing exosporium layers were found to adhere better to Caco-2 colonic cells and germinate more readily than spores with intact exosporiums.

The exosporium surrounds the next layer, the spore coat, which is commonly the outermost layer of spores in many other spore-forming bacteria. Despite many structural similarities, *C. difficile* only shares less than 25% of similar spore coat proteins homologs with the well-studied *B. subtilis*. The *C. difficile*
spore coat is proposed to contain a dense and rigid polypeptide lattice consisting of many cysteine residues that form disulfide bridges under aerobic conditions. The spore coat is resistant to harsh chemicals and functions as a protective barrier to the underlying spore cortex, which is susceptible to lysozyme degradation. It is also a passageway for small molecules known as germinants to pass through into the inner spore layers.

The cortex is sandwiched between an outer and inner membrane. The cortex is comprised of cross-linked layers of peptidoglycan. The cortex plays an essential role in dehydrating the spore's core, which contributes to the spore's heat resistance. During germination, the process by which the outgrowth of a vegetative cell occurs, the cortex is degraded by hydrolytic enzymes from the core. The degradation of the cortex is necessary for the full rehydration of the core upon germination. Directly underneath the cortex is the germ cell wall which will become the cell wall of the bacterial cell following germination.

The spore’s inner membrane is a stiff lipid bilayer. For Bacillus and Clostridium species other than C. difficile, the inner membrane contains the germination receptors (Ger). C. difficile lacks a known Ger receptor, but it is proposed that its germination receptor is present at this layer. The low permeability of the rigid inner membrane also aids in the protection of DNA in the underlying core.

The innermost spore layer is the core which stores large amounts of dipicolinic acid (DPA) chelated with calcium ions (Ca\(^2+\)). DPA is suggested to be
responsibility for the spore’s heat resistance. The Ca\textsuperscript{2+}-DPA complex makes up 20-25% of the core’s dry weight. Also in the core are small acid-soluble proteins (SASPs) that are tightly bound to DNA. They have been linked with the spore’s chemical and UV resistance. SASPs make up approximately 10-20% of the core's dry weight. Moreover, the spore’s low water content (25-60% wet weight) also contributes to its resistance capabilities. In addition to housing the genetical material of the spore (DNA and RNA), the core also contains enzymes that are required for degradation of the outer spore layers during germination and outgrowth.

Endospore formation occurs in a cycle and is characterized by several key morphological steps (Figure 1.5). In B. subtilis, it can take approximately 3 hours for the first morphological change to occur after sporulation is inaugurated. However, C. difficile can take between 8 to 20 hours for the same sporulation stage to jumpstart. This variability may explain possible differences in sporulation rates among various C. difficile strains. First, the vegetative bacterial cell senses an environmental stressor (Stage I). As a way to preserve the cell’s genetic material, it begins to form the endospore. This first step is characterized by the formation of a septum on one side of the cell (Stage II). Following septum formation, the peptidoglycan in the septum begins to degrade and smaller side of the cell becomes engulfed to form a forespore (sometimes referred to as the prespore) (Stage III). The remainder of the cell is referred to as the mother cell. The mother cell produces several endospore-
specific components that aid in the maturation of the fully formed endospore. The mother cell synthesizes the core and cortex (Stage IV), then manufactures the germ cell wall, inner membrane, and outer membrane (Stage V). After the inner layers are formed, the spore coat and exosporium are deposited around the spore (Stage VI). Once all layers are constructed, the mother cell programs cell death and lyses, causing the mature endospore to be released and freed (Stage VII). Upon exposure to ideal conditions and activation by a germinant (discussed further in section 1.4), the endospore can germinate, resulting in outgrowth of a vegetative toxin-producing cell (Stage VIII). As a defense mechanism to protect its DNA, the bacterial cell can reenter the sporulation cycle to produce more endospores under stressful conditions.
The presence of spores can be confirmed by microscopic visualization.

One approach is the Schaeffer-Fulton endospore staining technique which uses the malachite green to stain spores and safranin to counterstain bacterial cells (detailed in section 2.2.4.1).\textsuperscript{59} It was designed in 1933 by Alice B. Schaeffer and MacDonald Fulton, both of whom were microbiologist at Middlebury College in Middlebury, Vermont. It is sometimes referred to as the Wirtz-Conklin method.
after the two bacteriologists who originally developed the technique in 1908. Schaeffer and Fulton slightly modified Wirtz and Conklin’s previous method by replacing osmic acid with heat as a fixating agent to provide a technique that was faster and more efficient than other methods used at that time. One of those methods was Dorner’s endospore staining technique, published in 1922, which used Ziehl-Neelsen Carbol Fuchsin (ZNCF) to penetrate into the spores, causing them to appear red, and 10% Nigrosine to stain the background of the slide black.

1.2.3 Toxin Production

Upon germination and outgrowth of *C. difficile* spores, toxins are produced and released. These toxins are responsible for the pathogenicity of CDI and are the determinants for disease. The two major *C. difficile* toxins are enterotoxin TcdA (*Clostridium difficile* toxin A) and cytotoxin TcdB (*Clostridium difficile* toxin B), both of which have been implicated in CDI symptoms such as diarrhea and PMC. Several strains of *C. difficile* are also capable of producing a third toxin known as CDT (*Clostridium difficile* transferase), which has been frequently implicated in severe cases of CDI. Non-toxigenic strains of *C. difficile* also exist naturally and do not cause disease.

TcdA and TcdB are among the largest bacterial toxins known with molecular masses of 308 kDa and 270 kDa, respectively. TcdA and TcdB are encoded by genes *tcdA* and *tcdB*, which are found in open reading frames
situated within the 19.6 kb pathogenicity locus (PaLoc) of the \textit{C. difficile} chromosome.\textsuperscript{65,66} Additionally, \textit{tcdA} and \textit{tcdB} genes have low GC content as does the entire \textit{C. difficile} genome.\textsuperscript{60} The toxins also have a high degree of similarity with 66\% homology overall.\textsuperscript{60}

During the early exponential phase of \textit{C. difficile} cell growth, toxin release levels are relatively low. At this point, TcdC, a negative regulator of toxin production, is expressed at high levels.\textsuperscript{60,67,68} The \textit{tcdC} gene, which encodes TcdC, lies downstream of \textit{tcdA} and \textit{tcdB} and is transcribed in the opposite direction.\textsuperscript{60,67,68} By the late exponential phase to stationary phase, TcdC levels decline and toxin production significantly increases.\textsuperscript{60,67,68} Another regulator, TcdD, encoded by the upstream \textit{tcdD} gene, has been suggested to be a positive regulator of toxin production.\textsuperscript{60,69}

TcdA and TcdB are both glucosyltransferases that inactivate intracellular GTPases (Rho, Rac, and Cdc42 proteins) within target cells.\textsuperscript{60,70} These GTPases are regulatory proteins of the actin cytoskeleton.\textsuperscript{60,71} Both \textit{tcdA} and \textit{tcdB} genes contain four key domains (from C-terminus to N-terminus): the combined repetitive oligopeptide repeat (CROP) domain, the putative translocation domain, the cysteine protease domain (CPD), and the glucosyltransferase domain (GTD). The CROP domain, or receptor-binding domain, binds to carbohydrate and protein receptors on the surface of the host epithelial cells. TcdA binds to glycoprotein 96 (gp96) and sucrase-isomaltase on the apical membrane of human colonocytes; whereas, TcdB binds to chondroitin sulfate
proteoglycan 4 (CSPG4) and poliovirus receptor-like 3 (PVRL3) on the surface of intestinal epithelium.\textsuperscript{60,70-73} Upon interaction with the host cell receptors, the toxins are internalized via endocytosis. The endosome acidifies, causing the toxins to partake in conformational changes that allow for their translocation into the cytoplasm. Once the toxins are internalized into the host cell, toxins are auto-catalytically cleaved by their CPD domain, thus activating GTD, the catalytic domain. GTD aids in the transfer of glucose from UDP-glucose to the target GTPases, rendering them inactive. The glycosylation of the GTPases triggers modification of the actin cytoskeleton and increased permeability of tight junctions, leading to epithelial cell damage, increased fluid secretion, mucosal necrosis, and apoptotic cell death.\textsuperscript{21,60} Increased epithelial permeability also leads to neutrophil infiltration and accumulation, which are the hallmarks of pseudomembranous colitis (PMC) (Figure 1.6).\textsuperscript{21,60,70}
The detection of TcdA and TcdB have long been paramount for diagnosing CDI. Immunoassays and cytotoxicity assays have been employed to detection the presence of toxins. Enzyme immunoassay (EIA) tests have quick turnover times and can detect the presence of free TcdA and TcdB in stool.\textsuperscript{74-76} Due to their low sensitivity, toxin EIAs are usually not used as stand-alone diagnostic tools.\textsuperscript{77,78} The cell cytotoxicity neutralization assay (CCNA) have been long considered as the “gold standard” for \textit{C. difficile} toxin detection.\textsuperscript{77-79} CCNAs take the filtrate of collected stool samples and test them against mammalian cell cultures; cell round up and necrosis are indicative of the presence of TcdB. Verification of \textit{C. difficile} toxin as the cause of cytopathic effects is determined by neutralization with specific antitoxin antibodies.\textsuperscript{78,79}
While highly sensitive and specific, the CCNA has a slow turnaround time and requires technical expertise. The nucleic acid amplification test (NAAT) uses real-time quantitative polymerase chain reaction (RT-PCR) to amplify *C. difficile* toxin gene fragments. However, this method does not account for the expression of active toxins.

CDT is a ribosyltransferase that was first discovered in *C. difficile* strain CD196 by Popoff et al. in 1988. It was found to exhibit ADP-ribosyltransferase function similar to *Clostridium botulinum* toxin C2 and *Clostridium perfringens* toxin E iota. As a binary toxin, it is composed of two separate proteins: CdtA, the enzymatic component, and CdtB, the binding component. CDT binds to the lipolysis-stimulated lipoprotein receptor (LSR) on the surface of intestinal epithelial cells. Similar to TcdA and TcdB, CDT is internalized via endocytosis. CdtB creates a small pore in the acidified endosome facilitating the release of CdtA into the cytosol. There, CdtA ribosylates G actin, collapsing the host cell’s actin cytoskeleton by inhibiting actin polymerization and promoting fibronectin microtubule elongation and protrusion. Furthermore, this fosters *C. difficile*’s adherence to the intestinal epithelium.

CDT has been associated with hypervirulent (severe disease-causing) *C. difficile* strains such as the epidemic strain BI/NAP1/027. In addition to TcdA and TcdB, BI/NAP1/027 strains are also capable of producing active CDT. Although many other factors account for the virulence of BI/NAP1/027 strains, it is possible that CDT has synergistic effects with TcdA and TcdB. More on
hypervirulence and BI/NAP1/027 strains will be discussed in section 1.3.5 and chapter 2.

1.3 *Clostridium difficile* Infection (CDI)

1.3.1 Disease Characteristics

*Clostridium difficile* Infection (CDI) is a primarily nosocomial disease caused by *Clostridium difficile*. It is predominately linked to patients who have had prior antibiotic use.\(^8^4,^8^5\) Individuals who are elderly or immunocompromised are also disproportionally affected and are more susceptible to disease.\(^2^1\) Use of antibiotics such as clindamycin can disrupt the natural colonic gut flora, thus removing the beneficial bacteria and allowing for the colonization of *C. difficile*. Due to the resistant nature of *C. difficile* spores, they can survive passage through the acidic stomach, the small intestine, and into the large intestine (colon).\(^3^8\) When in the intestines, the anaerobicity and nutrient-rich environment promotes the spores to germinate into toxin-producing vegetative cells that are capable of eliciting disease.

CDI symptoms can range from mild to severe disease. Mild disease is defined solely as the presence of diarrhea.\(^8^5,^8^6\) Moderate disease includes a repertoire of other symptoms such as abdominal pain, loss of appetite, fever, nausea, vomiting, GI bleeding, bloody stools, and weight loss.\(^8^5\) Severe disease includes the same symptoms associated with mild-to-moderate diarrhea with the addition of more serious conditions such as elevated white blood cell
counts, hypotension, high grade fever, pseudomembranous colitis (PMC), ileus (the reduction in peristaltic movement of stools through the GI tract), abdominal distention, altered mental status, and organ failure.\textsuperscript{85} PMC can lead to another grave condition called toxic megacolon, which renders the colon incapable of expelling gas and stool, potentially leading to rupture. Moreover, organ failure can ultimately lead to death.

1.3.2 Mode of Transmission

CDI is transmitted through an oral-fecal route (Figure 1.7). \textit{C. difficile} spores can be ingested through the oral cavity and shed through feces. In healthy individuals, indigenous gut bacteria will assist in the regulation of bile acids that aid in the prevention of spore germination.\textsuperscript{87-89} Thus, the spores that enter the body will exit the body as spores.\textsuperscript{90} However, in patients with depleted gut microflora, \textit{C. difficile} spores can germinate in the colon and cause CDI symptoms.\textsuperscript{73,87-89}
The spore’s resistant qualities may allow it to survive for long periods of time. Contaminated surfaces, instruments, and equipment such as counters, beds, and gloves can become reservoirs for *C. difficile* spores. Therefore, decontamination is critical in the prevention of CDI from spreading. Proper hand hygiene and glove wearing are initial preventative measures used in hospital-related settings as precautions when working with CDI patients. Some hospitals have also implemented infection control programs.\textsuperscript{85,91} Because *C. difficile* spores are resistant to alcohol-based cleaners, disinfectants that contain a
minimum of 5,000 ppm of chlorine are recommended to kill the spores. Isolation of patients may also be required to reduce transmission from person to person. A cohort studied showed that patients in double rooms are more likely to acquire CDI than patients placed in single rooms.\textsuperscript{85,92}

Traditionally, CDI has been considered a nosocomial disease. However, some studies have also isolated \textit{C. difficile} outside of the hospital setting in non-human reservoirs. \textit{C. difficile} has been found in food as well as domestic animals.\textsuperscript{93-95} This could be one possible mode of transmission in the community-acquired CDI setting.

For some individuals, \textit{C. difficile} is indigenous rather than acquired through transmission.\textsuperscript{10,96,97} These individuals are often asymptomatic \textit{C. difficile} carriers and have the presence of other intestinal microbes that keep \textit{C. difficile} in check to manageable quantities. Asymptomatic carriers have also been shown to be less susceptible to CDI even under antibiotic therapy. This may possibly be attributed to their natural immune system. A study by Sanchez-Hurtado et al. suggested that carriers have increased antibody response to \textit{C. difficile}.\textsuperscript{98} Another study done by Kyne, Wann, Qamar, and Kelly showed that higher production of the antibody immunoglobulin G (IgG) against \textit{Clostridium difficile} toxin A helps confer immunity.\textsuperscript{99} Nevertheless, asymptomatic carriers may still pose a risk for transmitting \textit{C. difficile} to vulnerable individuals.\textsuperscript{97}
1.3.3 Establishment of Infection

There are three steps at the host level that enable the establishment of CDI: ingestion of spores, susceptibility to CDI due to antibiotic exposure, and loss of a natural protective barrier in the GI tract. Infection begins with the ingestion of *C. difficile* spores from the environment. In most archetypal CDI models, the host must become predisposed to CDI by antibiotic usage. Prior to antibiotic treatment, the gut flora is naturally in symbiosis (Figure 1.8). Antibiotics disrupt the natural microbiota within the GI tract causing dysbiosis (Figure 1.8). The loss of the natural protective barrier in the gut allows for *C. difficile* overgrowth and accumulation (Figure 1.8). Moreover, the depletion of beneficial gut microbes results in a crucial loss in the regulation of various factors involved in inhibiting the establishment of infection such as germination inhibition.
At the spore level, there are also three steps to establishing infection: the entrance of the spore into the host, germination of the spore, and production of toxins. Spores are the vehicle for infection. Although they do not cause disease symptoms, they are the infecting agents that enter the host and set the foundation for infection. Once the spores pass the acidic stomach, the favorable environment in the small intestine along with the presence of key germinants, in the form of bile salts and amino acids, promote spore germination.\textsuperscript{49,52,87,89,100} While the spore prepares for the outgrowth of vegetative cell, the spore travels to the large intestine (colon). There, the resultant metabolically active vegetative cells produce toxins, which are the determinants for disease. The internalization of the toxins eventually recruits...
neutrophils that cause an inflammatory response, giving rise to diarrheal symptoms and pseudomembranous colitis. Sporulation by the vegetative cells is also localized to the colon.

1.3.4 Current Treatment Options

CDI is usually instigated by broad-spectrum antibiotics used to treat other illnesses. Patients who are on cephalosporins, fluoroquinolones, and clindamycin are at even higher risk for contracting CDI.\textsuperscript{101} Once CDI is diagnosed, timely treatment is crucial. Ironically, CDI patients usually must stop taking those antibiotics to begin taking CDI-specific antibiotics. Conventionally, the antibiotics metronidazole and vancomycin have been used to treat CDI.

Metronidazole (trade name Flagyl) is a nitroimidazole antibiotic that is active against anaerobic bacteria (Figure 1.9). Due to its small size and low molecular weight, it can passively diffuse through the \textit{C. difficile} cytoplasm. Metronidazole acts by stealing electrons from bacterial electron transfer proteins flavodoxin and ferredoxin. This results in a nitroso free radical that hinders nucleic acid synthesis. Metronidazole is used to treat mild-to-moderate CDI. However, metronidazole is usually avoided in prolonged treatment as long-term use has been linked with neurotoxicity and hepatotoxicity.\textsuperscript{86,102} Also, resistance to metronidazole has previously been reported and can arise from factors such as slow drug activation, increased drug efflux, and horizontal gene transfer of nitroimidazole resistant genes.\textsuperscript{86,103}
For more moderate-to-severe cases of CDI, vancomycin is usually the drug of choice. Vancomycin (trade name Vancocin) is a cell wall inhibitor that targets mainly Gram-positive bacteria by preventing the synthesis of peptidoglycan (Figure 1.9). It forms hydrogen bonds with the terminal D-alanyl-D-alanine residues of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) peptides of the peptidoglycan layer. Vancomycin also prevents spore outgrowth. As a large hydrophilic molecule, vancomycin is poorly absorbed from the intestines. Therefore, in order to reach the site of infection (the GI tract), vancomycin must be administered orally for CDI cases. Unlike metronidazole, C. difficile has not yet been reported to show resistance to vancomycin. However, vancomycin is much more expensive than metronidazole.

Vancomycin can be used in conjunction with metronidazole to treat CDI recurrences, thought it may not be successful in preventing them. Because metronidazole and vancomycin both target a broad range of bacteria, they can suppress the growth of the intestinal microbiota, resulting in a high frequency of relapse. In recent years, a newer antibiotic called fidaxomicin (trade name Dificid) has been used in metronidazole- and vancomycin- failed treatment (Figure 1.9). Fidaxomicin was originally isolated from Dactylosporangium aurantiacum subspecies hamdenesis and is a macrocyclic antibiotic. Fidaxomicin prevents the opening and closing of the DNA/RNA clamp responsible for the initiation of transcription. Although
Fidaxomicin shows minimal disruption to the indigenous gut microflora, one caveat is that it is relatively ineffective against recurrent CDI. Despite this, fidaxomicin has been associated with lower rates of recurrence.

Several novel treatment options are available for patients suffering from CDI. For more extreme cases of CDI, fecal microbiota transplantation (FMT) is an alternative approach to treating multiply-recurrent CDI. FMT is the introduction of healthy donor stools into the colon of CDI patients. This method is used to restore the diverse gut microbiota that was killed off by antibiotics and reinstate resistance to C. difficile colonization. FMT is typically administered via colonoscopy, endoscopy, or through an enema, but can also be taken as a frozen oral pill. Although success rates are generally high (86-99%), the procedure can be aesthetically unappealing. The perceived concern for...
transmission of infectious agents of the donor stools has also been a topic of concern.\textsuperscript{85,115} However, a 2011 review found that there were no reported infection transmission reports in over 370 published studies.\textsuperscript{118} Regardless, donor stools undergo careful selection and rigorous screening prior to being used in treatment, which increases the cost of the treatment.\textsuperscript{119}

Figure 1.10. Representation of fecal microbiota transplantation (FMT). Donor stools are carefully screened and selected for use in FMT (Step 1). The stools are blended, strained, then stored in a freezer (Step 2). Prior to treatment, the stools are portioned (Step 3) and administered to the patient via colonoscopy/endoscopy tube, enema, nasogastric tube, nasoduodenal tube, orogastric tube, or oral capsule (Step 4).

Other novel treatments include the use of probiotics, vaccines, and antitoxins. Like FMT, probiotics introduces “good” microorganisms into the GI tract of CDI patients (Figure 1.11). Unlike FMT, it usually only includes a few specific combinations of microbial species. Common microorganisms included
in probiotics are *Saccharomyces boulardii, Lactobacilli, Clostridia, Streptococci,* and *Bifidobacteria.* *Lactobacilli* and *Bifidobacteria* are ordinarily found in yogurt and dairy products, respectively. Probiotics are usually administered in tablet form. Several meta-analyses have shown probiotics to be useful in treating Irritable Bowel Syndrome (IBS) and CDI.\textsuperscript{120,121} A few sporadic cases of probiotic therapy have reported bloodstream infections due to bacteremia and fungemia in immunocompromised patients.\textsuperscript{122,123} This brings up the concern of probiotic safety evaluation as they are currently not overseen by a major regulatory entity.\textsuperscript{124,125} Probiotic therapy, along with other innovative treatment options such as vaccines and antitoxins, are still relatively new and theirs benefits with treating CDI are still under investigation.\textsuperscript{126-129}
The CDI Dilemma

Previously, CDI has been associated with those who are in a hospital-related setting or those who are elderly and/or immunocompromised. However, in the past decade, CDI has been on the rise in both hospital and community settings. CDI incidences rose due, in part, to emergence of hypervirulent strains. Hypervirulent strains, such as the previously introduced BI/NAP1/027 strains, exhibit a wide variety of characteristics that attribute to their “hypervirulence.” Hypervirulent strains can exhibit increased sporulation rates, increased toxin production, or sometimes both, which can contribute to more
severe diarrhea and disease progression, increased recurrence, and higher mortality rates.

The emergence of new strains has also been noted as the probable cause of the growing problem of community-acquired CDI. As briefly discussed in section 1.3.2, food and domestic animals can be a possible source of CDI transmission in the community setting. In addition, transmission through asymptomatic carriers and healthcare workers have also been reported. However, many community-acquired CDI cases have shown an absence of classic risk factors for CDI such as antibiotic exposure, elderly age, comorbidity/immunosuppression (e.g., cancer, HIV), and a clinical setting. The multitude of discovered C. difficile strains make it increasingly difficult to treat CDI with standard methods. Some strains are also specific to certain settings and geographical regions. PCR-ribotyping, which is a method of bacterial identification based on the similarities of polymorphisms in the 16S rRNA gene, allows researchers to categorize these different C. difficile strains into groups, making similarities and differences between strains easier to identify.

Treatment of CDI and other afflictions with broad-spectrum antibiotics can also produce a viscous cycle of antibiotic use, which further predispose individuals to CDI recurrences. With CDI recurrences of up to 25%, standard treatments are rendered less effective. As a multi-faceted problem, new avenues of treatment for CDI need to be explored. The dilemma with CDI is that
it is an antibiotic-associated disease that is commonly treated with antibiotics. Therefore, finding prophylactic/preventative methods are critical.

1.4 Spore Germination and Inhibition

1.4.1 Germination

Spore germination is necessary for the establishment of CDI. When the C. difficile spore is exposed to ideal conditions, such as the anaerobic, nutrient-rich GI tract, germination can occur. This phenomenon begins with the hydration of the spore. As water enters the spore, large amounts of Ca$^{2+}$-DPA complex are released from the spore. Hydrolytic enzymes are also released from the spore core to degrade the cortex and spore coat. The destruction of the cortex is necessary for the full hydration of the anhydrous core.$^{45,48,142,143}$ SASPs are hydrolyzed into smaller amino acids that are used for the outgrowth of the vegetative cell. The germ cell wall also becomes the cell wall of the bacterial cell.

Spore germination is expedited by the presence of germination promoters called germinants. When the germinants bind to the spore, the germination process is irreversible.$^{48,88}$ In most Bacillus and Clostridium species, spore germinants include amino acids, saccharides, nucleosides, and ions.$^{45,49}$ In contrast, C. difficile spore germinants are specific bile acids that are initially produced by the liver or are later modified in the intestines.$^{87}$ These germinants are proposed to bind to Ger receptors on the spore.$^{54,144}$ C. perfringens possess
three germination-specific subtilisin-like serine proteases, CspA, CspB, and CspC, that act by cleaving lytic enzymes responsible for degrading the spore cortex.\textsuperscript{145-147} In \textit{C. difficile}, CspA and CspC are predicted to be catalytically dead. However, a 2013 study by Francis \textit{et al.} suggested that mutations in CspC alter the spore’s response to bile salts.\textsuperscript{147} Identification of a putative \textit{C. difficile} germination receptor is currently being studied.

1.4.2 The Role of Bile Salts on Spore Germination and Inhibition

Past studies have showed that bile acids play an instrumental role in the promotion and inhibition of \textit{C. difficile} spore germination.\textsuperscript{87-89,100} Bile acids are steroid compounds that are produced in the liver and stored in the gallbladder. Upon released from the gallbladder, their primary function is the emulsify (physically separate) larger fat aggregates to aid in lipid digestion and reabsorption in the small intestines.

The synthesis of bile acids begins in the liver with cholesterol (Figure 1.12). In the liver, cholesterol is converted to the primary bile acids chenodeoxycholate and cholate through several enzymatic steps.\textsuperscript{89,148,149} With the addition of the amino acid glycine or the amino acid derivative of cysteine called taurine, the primary bile acids become conjugated bile acid (also known as bile salts). For chenodeoxycholate, the bile salt derivatives are glycochenodeoxycholate and taurochenodeoxycholate. For cholate, the bile
The liver secretes the synthesized bile acids as bile salts. Once the gallbladder releases bile containing bile salts into the intestines, the gut microbiota takes over the modification of the bile salts (Figure 1.12). In the upper ileum, bile salt hydrolases, produced by many intestinal microbes, deconjugate the bile salts by cleaving their amino acid side chains and converting them back into primary bile acids. When unabsorbed bile acids reach the lower ileum, indigenous clostridial species such as C. scindens dehydroxylates the primary bile acids via 7α-dehydroxylase. The products are lithocholate (from chenodeoxycholate) and deoxycholate (from cholate). These are known as secondary bile acids. Most of the bile acids are reabsorbed through active transport by ileal mucosal cells and transported back into portal blood by enterohepatic circulation. Interestingly, while chenodeoxycholate is very readily absorbed into the gut, its dehydroxylated derivative lithocholate is poorly absorbed. Accumulation of lithocholate in the colon revealed carcinogenic properties in the early stages of colon cancer.
Bile acids also have a secondary role of regulating *C. difficile* spore germination, though they are at the mercy of the indigenous gut microflora that are responsible for maintaining them through conjugation and hydrolysis. Taurocholate, and to lesser extents, cholate and glycocholate, have been
found to be natural germinants of *C. difficile* spores; whereas, chenodeoxycholate was discovered to be a natural germination inhibitor (Figure 1.12). Taurocholate also requires a co-germinant, the amino acid glycine, to activate *C. difficile* spore germination. Although glycine alone does not promote germination, it acts synergistically with taurocholate by increasing cooperative binding affinity to the spore. In a state of symbiosis such as in a healthy digestive tract, native bacteria keep the amounts of bile acids present in the gut in check. Hence, there are less germinants and more inhibitors present as bile acids move further down the GI tract (Figure 1.13). Due to the careful balance of bile acid content, the *C. difficile* spores that enter the gut do not germinate and are shed in feces. However, in patients who have depleted gut microbiota induced by antibiotics, a state of dysbiosis is present. Therefore, the remaining microorganisms are much less efficient at regulating the bile salts. Consequently, there is a buildup of germinants and lower presence inhibitors are present further down the gut (Figure 1.13). *C. difficile* spores entering the body are more likely to germinate around the increased attendance of germinants, causing CDI symptoms.
1.4.3 Targeting the Germination Pathway

Due to spore germination being a pivotal step in CDI establishment, anti-germination therapy has been proposed as a CDI prophylactic target. Past studies in the Abel-Santos Laboratory have proposed the production of synthetic bile salt analogs to compensate for the imbalance in key natural bile salts due...
to antibiotic-associated microflora disruption. It was hypothesized that modifying
the side chain of cholate (cholic acid) would result in possible competitive
inhibitors of cholate derivatives for the binding to the C. difficile spore (Figure
1.14).

One successful synthetic bile salt analog called CamSA is a cholate with a
meta-aminobenzene sulfonic acid side chain (Figure 1.15). Through kinetic
analysis, CamSA was shown to be a 275 times better binder to the spore than
taurocholate and was found to be a five times better germination inhibitor than
chenodeoxycholate. CamSA was found to be a germination inhibitor in strain
630 and VPI 10463 in vitro.\textsuperscript{157} CamSA had half maximal inhibitory concentration
(IC\textsubscript{50}) of 58.3 µM against strain 630 spores.\textsuperscript{100} In mice challenged with C. difficile
strain 630, CamSA was able to prevent CDI with a single 50 mg/kg dose.\textsuperscript{157}
Furthermore, CamSA showed no observable toxicity to mice.\textsuperscript{90,157} In studies with
simulated gastric and intestinal juices, CamSA remains stable.\textsuperscript{90} Despite being
highly susceptible to CDI, hamsters given C. difficile strain 630 with CamSA showed reduced CDI symptoms when coupled with vancomycin therapy.

Following these initial CamSA studies, the effects of CamSA against other strains of C. difficile needed to be uncovered. After preliminary testing of CamSA with the hypervirulent BI/NAP1/027 strain R20291, CamSA was unable to prevent spore germination *in vitro*. Although CamSA was infective against strain R20291 *in vitro*, it’s *in vivo* effects were still of interest. It is also possible that CamSA is potent against other strains of C. difficile that were not yet tested. To find a new potent germination inhibitor, over 200 CamSA analogs were screened. Of the compounds tested, one compound called 07C was discovered to inhibit spore germination in both germination in strain 630 as well as strain R20291 both with less than 10 μM concentrations (Figure 1.15). Other screened compounds did not display as promising preliminary results as 07C, though many more compounds are still being screened in the Abel-Santos Laboratory.
1.5 Specific Aims of This Study

The first specific aim of this study is to test synthetic bile salt analog compounds against various *C. difficile* strains *in vitro*. Prior to this study, CamSA analogs were pre-screened *in vitro* via germination assays that helped identify each compound's potential to inhibit spore germination. The compound that possessed the lowest inhibitory concentration against the hypervirulent *C. difficile* strain R20291 was the cholic acid aniline derivative 07C. Therefore, CamSA and 07C were chosen and tested as leading potential germination inhibitors for this specific aim. The germination assay also allowed for compound potency testing to investigate whether the compounds are inhibitors or promotors of spore germination for each *C. difficile* strain at varying concentrations.

From the *in vitro* analyses, testing of the compounds against various strains *in vivo* was the next specific aim. The mice model was used to observe animals...
for signs for CDI. The mice model also allowed for visual observation of more subtle differences between infection symptoms of CDI caused by the different strains. Mice symptoms severities were scored via a CDI rubric. Mice that received spores only were compared to those that received spores and compound treatment.

The hypothesis of this study is that the behavior of CamSA and 07C against the various C. difficile strains in vitro may give insight into their ability to prevent CDI in the animal model. Moreover, prophylactic treatment of CDI through these CamSA analogs can act as molecular probes to target C. difficile’s spore germination pathway.
CHAPTER 2

IN VITRO STUDIES:

GERMINATION PROFILES OF CLOSTRIDIUM DIFFICILE CLINICAL ISOLATES

2.1 Introduction

In 2011, Tenover et al. typed 350 toxigenic strains of C. difficile from seven laboratories in the United States and Canada. The strains were organized by three molecular typing techniques: PCR-ribotyping, pulsed-field gel electrophoresis (PFGE), and restriction endonuclease analysis (REA). Of the strains that were typed, approximately 70% were of known PCR-ribotypes, 54% were of known PFGE groups, and 70% were of 1 of 8 common REA groups. Some isolates typed using one method also overlapped with clusters from the other typing methods, while others did not. Therefore, using more than one typing method can give a more comprehensive outlook on the epidemiology of the rising diversity of C. difficile strains and their roles in CDI.

Some categories of isolates may display other unique characteristics such as the higher prevalence in specific geographical regions. For example, a Southern Taiwanese study found that C. difficile ribotypes 017 and 078 were predominant among toxigenic clinical isolates from stools collected from medical wards of the district hospital in the area. Similarly, rising prevalence of C. difficile ribotype 078 was found in hospital settings in Europe and the United States. Ribotype 078 isolates found in Canada were, however, more prevalent among cattle and swine than in humans.
Some groups of isolates also exhibit resistance to certain anti-microbial agents and genome alterations that confer hypervirulence. Of interest are the C. difficile BI/NAP1/027 strains. BI refers to the BI group based on REA, NAP1 is the North American pulsed-field type 1 based on PFGE, and 027 is the PCR-ribotype. BI/NAP1/027 strains were isolated from several hospital facilities around North America and Europe. Strains of PCR-ribotype 027 have been linked with fluoroquinolone antibiotic resistance.\textsuperscript{83,161} Although fluoroquinolone antibiotics are not commonly prescribed to treat CDI, BI/NAP1/027’s resistance to them have been thought to be a factor in its epidemic spread and hypervirulence.\textsuperscript{83,162} One PCR-ribotype 027 strain called R20291 caused recent CDI epidemics in Europe and North America.\textsuperscript{133} Strain R20291 as well as other PCR-ribotype 027 strains have been deemed “hypervirulent” as they were involved in cases of severe diarrhea, high mortality rates, and high recurrences.\textsuperscript{133,163-165} Moreover, PCR-ribotype 027 isolates have been found to have 234 additional genes compared to type strain 630 (epidemic type X, PCR-ribotype 012).\textsuperscript{166} A deletion at position 117 of the tcdC gene (responsible for repression of toxin A and B production) has been implicated as the cause of increased toxin production in BI/NAP1/027 strains.\textsuperscript{83,166,167}

As the vehicle for infection, studying how spores germinate is important to understanding how diverse strains cause CDI. One method used to explore spore germination is by exploiting their unique property of optical density loss.\textsuperscript{168-170} Spores are optically dense structures. Upon hydration of the spore core and
degradation of the spore coat, the spore loses its natural refractility, and thus loses optical density. This can be observed by spectrophotometry.

By employing optical density methods, Heeg et al. mapped the germination profiles of spores from various C. difficile clinical isolates including from strain R20291. Although some strains displayed expected germination in the presence of the natural germinant taurocholate (TC) and germination inhibition in the presence of the natural inhibitor chenodeoxycholate (CDCA), some strains (R20291, 8085054, CDC 38, DH1834, and 7004578) interestingly displayed some germination in the presence of 2 mM CDCA when incubated with 0.1% TC in supplemented brain heart infusion (BHIS) (Table 2.1). In contrast, strain 05-1223-046, exhibited high germination when incubated with 0.1% TC in BHIS. Although no unusual germination pattern arose with strain 9001966, it was unable to replicate on BHIS media without TC supplementation. Due to their unique germination and growth profiles, seven of these strains, along with type strain 630, were used in this thesis study (Table 2.2).
### Table 2.1. Percentage loss of spore optical density of select *C. difficile* clinical isolates.

<table>
<thead>
<tr>
<th>Strain</th>
<th>0.1% TC</th>
<th>2 mM CDCA</th>
<th>BHIS</th>
</tr>
</thead>
<tbody>
<tr>
<td>9001966</td>
<td>47%</td>
<td>3%</td>
<td>11%</td>
</tr>
<tr>
<td>8085054</td>
<td>72%</td>
<td>13%</td>
<td>38%</td>
</tr>
<tr>
<td>CDC 38</td>
<td>42%</td>
<td>12%</td>
<td>6%</td>
</tr>
<tr>
<td>DH1834</td>
<td>58%</td>
<td>57%</td>
<td>27%</td>
</tr>
<tr>
<td>05-1223-046</td>
<td>74%</td>
<td>0%</td>
<td>38%</td>
</tr>
<tr>
<td>7004578</td>
<td>46%</td>
<td>30%</td>
<td>7%</td>
</tr>
</tbody>
</table>

Spore were heat treated at 60°C for 25 minutes, then incubated with either 0.1% TC in BHIS, 0.1% TC with 2 mM CDCA in BHIS, or BHIS only. Data from this table was obtained by Heeg et al. 2012.171

### Table 2.2. *C. difficile* clinical isolates used in this thesis study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>PCR-Ribotype</th>
<th>Country of Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>9001966</td>
<td>002</td>
<td>The Netherlands</td>
</tr>
<tr>
<td>630</td>
<td>012</td>
<td>Switzerland</td>
</tr>
<tr>
<td>8085054</td>
<td>014</td>
<td>The Netherlands</td>
</tr>
<tr>
<td>CDC 38</td>
<td>027</td>
<td>USA</td>
</tr>
<tr>
<td>DH1834</td>
<td>027</td>
<td>East of England, Ipswich, UK</td>
</tr>
<tr>
<td>R20291</td>
<td>027</td>
<td>Stoke Mandeville, UK</td>
</tr>
<tr>
<td>05-1223-046</td>
<td>027</td>
<td>Belgium</td>
</tr>
<tr>
<td>7004578</td>
<td>078</td>
<td>The Netherlands</td>
</tr>
</tbody>
</table>
Previously, CamSA has only been tested as an anti-germinant against strain 630. When incubated with 6 mM TC and 12 mM glycine, CamSA was able to prevent strain 630 spore germination with a half maximal inhibitory concentration (IC$_{50}$) of 58.3 μM.$^{100}$ In recent preliminary studies performed in the Abel-Santos Laboratory, CamSA was unable to prevent spore germination in the hypervirulent strain R20291 at concentrations up to 100 μM. Because of this drawback, the Abel-Santos Laboratory screened over 200 other bile salt analogs as potential anti-germinants against multiple C. difficile strains. From these screens, we found some anti-germinant candidates that had IC$_{50}$ values smaller than CamSA against strain R20291. The best anti-germinant, compound 07C, prevented spore germination in both strain 630 and strain R20291 at concentrations below 100 μM. The goal of this project is to examine the effects of CamSA and 07C on the spore germination of eight selected C. difficile strains. The compounds’ abilities to prevent spore germination in vitro will give insight into possible in vivo effects.

### 2.2 Materials and Methods

#### 2.2.1 Materials

C. difficile strains R20291, 9001966, 05-1223-046, CDC 38, DH1834, 7004578, and 8085054 were generously donated by Professor Nigel Minton at the University of Nottingham in Nottingham, United Kingdom. C. difficile strain 630
was purchased from the American Type Culture Collection (ATCC). Synthesized bile salt analogs were provided by Professor Steven M. Firestine at Wayne State University in Detroit, Michigan, or were previously synthesized in the Abel-Santos Laboratory.

2.2.2 C. difficile Sporulation

C. difficile cells from a stock culture, frozen in 25% glycerol in brain heart infusion (BHI) broth supplemented with 0.5% yeast, were streak plated onto BHI agar supplemented with 2% yeast extract, 0.1% L-cysteine-HCl, and 0.05% sodium taurocholate (BHIS) to yield single-cell colonies. After 48 hours, a single colony was inoculated into BHI broth supplemented with 0.5% yeast extract and incubated for 48 hours. The inoculated broth was then spread plated onto BHI agar prepared as described above. Inoculated plates were incubated for 7 days at 37°C in an anaerobic chamber (10% CO₂, 10% H₂, 80% N₂).

2.2.3 C. difficile Spore Harvest and Purification

Prior to harvesting spores, inoculated plates were flooded with ice-cold deionized (DI) water. Cells and spores were then harvested by scraping bacteria colonies from the plates. The harvested cells and spores were pelleted via centrifugation at 8,000 × g for 5 minutes. The mixture was then resuspended in DI water and pelleted again. This washing step was repeated twice more. After the three washing steps, the mixture was centrifuged through a 20% (5 mL) to 50%
(10 mL) HistoDenz™ gradient at 18,200 × g for 30 minutes with no brake (Figure 2.1). As dense structures, only spores can penetrate through the 50% HistoDenz™ layer to form a thick pellet at the bottom of the centrifuge tube, while vegetative cells and small fragments of incidentally scraped off agar are lighter, consequently remaining above the 20% HistoDenz™ layer. The pelleted spore was then transferred to a clean centrifuge tube where it is washed five more times before being stored in DI water at 4°C. To determine spore purity, selected samples were stained using Schaeffer-Fulton endospore staining method or were visualized via phase contrast microscopy (discussed further in section 2.2.4). Spore preparations used were greater than 95% pure after centrifugation through the HistoDenz™ gradient (Figure 2.2).

Figure 2.1. Spore purification via HistoDenz™ gradient.
C. difficile spores and cells were visualized before and after spore harvest via light microscopy. Two microscopic visualization methods were used to determine the presence and number of spores: Schaeffer-Fulton endospore staining method and phase contrast microscopy.

2.2.4.1 Endospore Staining Technique

The Schaeffer-Fulton method is an endospore staining technique that utilizes malachite green and safranin to differentiate endospores from vegetative cells (Figure 2.3). In the Schaeffer-Fulton method, a small inoculum of bacteria is placed in a glass microscope slide and mixed with a drop of DI
water using an inoculating loop. The inoculum is air dried, then heat fixed to the slide by waving the slide over a flame three to five times. A small porous paper is then placed over the slide and is drenched with the primary stain malachite green. The endospore is forced to uptake malachite green by heat from an open flame for 5 minutes. The heat acts as a mordant to fix the malachite green to the spores. The excess malachite green is washed from the slide with DI water for 10-20 seconds to decolorize vegetative cells. Vegetative cells are then counterstained with safranin for 30 seconds. Endospores and vegetative cells can be visualized under a light microscope at 1000x magnification. Endospores will appear green and vegetative cells appear pink (Figure 2.4).
Figure 2.3. Representation of Schaeffer-Fulton endospore staining technique. Heat-fixed bacteria are stained with the primary stain malachite green (Step 1). Heat is applied to the bacteria while malachite green is continuously added for 5 minutes to allow the dye to penetrate spores and become retained (Step 2). Excess dye is washed with water for 10-20 seconds resulting in the decolorization of vegetative cells (Step 3). The secondary stain safranin is added for 30 seconds to counterstain vegetative cells (Step 4). Vegetative cells will appear pink while spores appear green under light microscopy.
Endospores can also be visualized using phase contrast microscopy. This method does not require staining, but requires a specialized phase contrast lens to detect differences between vegetative cells and spores. Due to their high refractive index, dormant spores show as bright structures, while germinated spores and vegetative cells appear as dark structures. A small inoculum of bacteria is placed on a glass microscope slide and mixed with a drop of DI water using an inoculating loop as described in 2.2.4.1. A glass cover slip is placed over the bacteria sample. The slide is viewed under the 1000x magnification phase contrast microscope lens. Under the phase contrast lens,
endospores will appear to glow with a halo of light surrounding them, which is caused by diffracted light passing through the structures (Figure 2.5).

Figure 2.5. Phase contrast microscopy of C. difficile strain 630 spores. Only spores appear to have the characteristic surrounding glowing halo.

2.2.5 Preparation of Spore Germination Assay

Spores were washed three times with DI water, then heat shocked at 68°C for 30 minutes. The heat shocking process both kills vegetative cells that may be present within the spore suspension and heat-activates spores so that they become more responsive to germinants. After heat-shock, spores were washed an additional three times. Heat-shocked spores were resuspended into germination buffer (0.1 M sodium phosphate buffer supplemented with 0.5% sodium bicarbonate and adjusted to pH 6.0) to reach an optical density at 580
nm (OD$_{580}$) of 1.00. Optical density was measured using a Thermo Scientific Spectronic Genesys™ 10 Bio UV-Vis spectrophotometer using germination buffer as a blank.

Stock solutions were prepared in either DMSO or H$_2$O. A 120 mM sodium taurocholate solution was prepared in DMSO. A 480 mM glycine solution was prepared in H$_2$O. Other bile salt analogs were also prepared at various concentrations in DMSO.

The spore germination assays were performed in 96-well plates (Figure 2.6). All experiments were done in triplicate. As negative germination controls, spores were treated with neat DMSO. As positive germination controls, spores were treated with 6 mM of sodium taurocholate and 12 mM of glycine. To test for spore germination inhibition, bile salt analogs were added at different concentrations to spores. Co-germinant solutions were then pipetted into wells containing the experimental groups for a final concentration of 6 mM of sodium taurocholate and 12 mM of glycine.

Following the addition of compound, 180 μL of spores in germination buffer (OD$_{580} = 1.00$) were added to each well. The final volume in each well was 200 μL. Optical density over time was read by a Labsystems iEMS Reader MF plate reader using Ascent™ software or a Tecan Infinite M200 plate reader using Tecan i-control™ software.
2.2.6 Analysis of Spore Germination and Inhibition Properties

Data obtained following experimental run were transferred to Microsoft Excel for preliminary data analysis. Optical density change over time was graphed (Figure 2.7). Optical density readings that did remain the same (at \( \text{OD}_{580} = 1.00 \)) over the course of the experiment indicated spores that did not germinate.\(^87,88,168,169\) In contrast, decreasing optical density correlated with spore germination.\(^87,88,168,169\)

Preliminary data analysis allowed for the inhibitory concentration ranges of the bile salt analog compounds against each \( C. \ difficle \) strain to be
determined. Following determination of inhibitory concentration ranges, intermediate concentrations (minimum of 9 values) were used to determined percent (%) germination by comparing the slope from the germination curves of each concentration of bile salt analog to the slope from the germination curve of the positive germination control. The slopes were taken from the linear portions of each concentration curve from the germination assay kinetic graphs at around the same timeframe (approximately before 50-60 minutes elapsed time) (Figure 2.7). Germination kinetic graphs containing the concentrations of bile salt analogs used to construct dose-dependent curves to determine IC$_{50}$ values are included in Figures S1-S6, Appendix.

The bile salt analog concentrations with their respective percent germination values were then transferred to SigmaPlot Version 11 or SigmaPlot Version 13 and fitted with the four-parameter logistic function to obtain dose-dependent curves used to determine the IC$_{50}$ values for anti-germinant compounds.$^{179,180}$ Adjusted R$^2$ values from the regression analyses were above 0.95. IC$_{50}$ values represent the amount of compound required to reduce spore germination rate to half the maximal value and are used to compare inhibitory potency of bile salt analogs.$^{100,179,180}$ Compounds that had IC$_{50}$ values under 100 μM were considered active inhibitors, whereas, compounds that had IC$_{50}$ values above 100 μM were deemed inactive.
When necessary, polymerase chain reaction (PCR) was used to amplify specific segments of genomic DNA. DNA band analysis via 1% agarose gel electrophoresis confirmed the presence or absence of *C. difficile*. Specific forward and reverse primers were used to detect *C. difficile* and consider the possibility of *C. sordellii* contamination (another *Clostridium* species used in the

**2.2.7 C. difficile PCR Confirmation**

The blue line represents the negative germination control in which no germination occurs (no loss in optical density). The orange line represents the positive germination control in which spores are germinating (losing optical density) over time. The area in the red box represents the linear portion of the curve that will be used to determine the slope of the positive germination control. The ratio of experimental group slope to the positive germination control slope will determine percent spore germination.
Abel-Santos Laboratory). Primers were ordered from Integrated DNA Technologies (IDT). Primers, 2.5 μL of each, were added to 1 μL of genomic DNA, then mixed with 6.5 μL of water and 12.5 μL of Lucigen EconoTaq® PLUS 2x Master Mix containing EconoTaq DNA polymerase, dNTPs, MgCl₂, a PCR enhancer, and reaction buffer, for a final volume of 25 μL. DNA was denatured at 94°C annealed at 50°C, and extended at 68°C in a Bio-Rad MyCycler™ Thermal Cycler.

Following amplification, 5 μL of each sample of PCR contents [mixed with 1 μL of 6x loading dye] was added to individual wells of a 1% agarose gel prepared with 1x TAE (Tris-acetate-EDTA) buffer and stained with 0.5 μg/mL ethidium bromide (EtBr) to act as a fluorescent tag. Gel electrophoresis was run for 20 minutes at a voltage of 135 V, current of 500 mA, and wattage of 250 W. DNA bands were analyzed using the UVP VisionWorks®LS Analysis Software and the UVP EC3 Imaging System. A Promega BenchTop 1kb DNA ladder was used for reference markers.

2.3 Results and Discussion

2.3.1 Germination Profile of strain 630

Both CamSA and 07C were able to prevent germination of C. difficile strain 630 spores. The IC₅₀ value of CamSA against strain 630 was previously determined to be 58.3 μM. Therefore, this germination assay was only used to confirm that CamSA could prevent spore germination at around that same
At 50 μM CamSA, 31% of spore germination was inhibited. 07C was found to be a better germination inhibitor than CamSA by 7-fold against strain 630. Spores were treated with 6 mM taurocholate and 12 mM glycine and added to various concentrations of 07C (Figure 2.8). At 6.25 μM 07C, 39% of spore germination was inhibited. At 50 μM 07C, spore germination was mostly inhibited. The IC<sub>50</sub> of 07C against strain 630 was determined to be
8.19 μM by regression analysis of a dose-dependent curve (Figure 2.9). The adjusted $R^2$ for this curve was 0.9654.

![Graph showing dose-response curve](image)

Figure 2.9. IC$_{50}$ calculation for 07C against *C. difficile* strain 630 spores. The graph represents the dose-response curve of strain 630 spores germinated with fixed concentrations of taurocholate (6 mM) and glycine (12 mM) added to various concentrations of 07C. The IC$_{50}$ value of 07C obtained from this regression is 8.19 μM. The germination assay kinetic graph that includes the 07C concentrations used to determine this IC$_{50}$ value can be found in Figure S1, Appendix.

### 2.3.2 Germination Profile of strain R20291

The germination assay for the hypervirulent strain R20291 displayed unique results. Contrary to expectation, CamSA was unable to prevent germination of strain R20291 spores. At 50 μM CamSA, spores germinated at about the same
rate as the positive germination controls containing only taurocholate and
glycine (Figure 2.10). Moreover, up to 1000 μM CamSA, spore germination was
comparable to the positive germination control. It is possible that CamSA might
be able to prevent loss in optical density of strain R20291 spores, but may require
higher concentrations in the millimolar range.

Figure 2.10. Effects of germinants and inhibitors on the germination kinetic behavior of C.
difficile strain R20291 spores. Spores were resuspended in germination buffer and treated with
neat DMSO (○) or treated with a fixed concentration of taurocholate (6 mM) and glycine (12
mM) added to final concentrations of 0 μM of bile salt analog compound (Δ), 50 μM CamSA
(■), 1.5625 μM 07C (▲), 6.25 μM 07C (●), and 25 μM 07C (●●). In actuality, more concentrations
of bile salt analogs were used and data was collected every minute for 120 minutes (Figure
S2, Appendix). For clarity, select concentrations are represented and data at five minute
intervals are shown. The data points indicate the means from three independent measures (n
= 3) and the error bars signify standard deviation from the mean.
Although CamSA was unable to prevent germination of strain R20291 spores, 07C prevented spore germination even at low micromolar concentrations (Figure 2.10). At just 1.5625 μM 07C, 43% of spore germination was inhibited. At 25 μM 07C, spore germination was inhibited by 87%. Regression analysis from a dose-dependent curve revealed an IC$_{50}$ of 1.92 μM for 07C against strain R20291 with an adjusted R$^2$ of 0.9922 (Figure 2.11).

![Figure 2.11. IC$_{50}$ calculation for 07C against C. difficile strain R20291 spores. The graph represents the dose-response curve of strain R20291 spores germinated with fixed concentrations of taurocholate (6 mM) and glycine (12 mM) added to various concentrations of 07C. The IC$_{50}$ value of 07C obtained from this regression is 1.92 μM. The germination assay kinetic graph that includes the 07C concentrations used to determine this IC$_{50}$ value can be found in Figure S2, Appendix.](image)

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2.3.3 Germination Profile of strain 9001966

Similar to strain R20291, strain 9001966 spores were able to germinate in the presence of CamSA (Figure 2.12). Up to 100 μM CamSA, loss of optical density remained nearly the same as the positive germination control. Since the cutoff for inhibition activity was set at 100 μM, CamSA was deemed inactive against strain 9001966.

Figure 2.12. Effects of germinants and inhibitors on the germination kinetic behavior of C. difficile strain 9001966 spores. Spores were resuspended in germination buffer and treated with neat DMSO (○) or treated with a fixed concentration of taurocholate (6 mM) and glycine (12 mM) added to final concentrations of 0 μM of bile salt analog compound (△), 50 μM CamSA (■), 6.25 μM (▲), 25 μM 07C (★), and 50 μM 07C (●). In actuality, more concentrations of bile salt analogs were used and data was collected every minute for 120 minutes (Figure S3, Appendix). For clarity, select concentrations are represented and data at five minute intervals are shown. The data points indicate the means from three independent measures (n = 3) and the error bars signify standard deviation from the mean.
Germination inhibition of strain 9001966 spores required higher concentrations of 07C than strain R20291. Like, strain 630, 07C was able to inhibit spore germination at similar concentrations (Figure 2.12). At 6.25 μM 07C, 42% of spore germination was inhibited. The IC₅₀ for 07C against strain 9001966 was found to be 7.60 μM with an adjusted R² of 0.9915 (Figure 2.13).

Figure 2.13. IC₅₀ calculation for 07C against C. difficile strain 9001966 spores. The graph represents the dose-response curve of strain 9001966 spores germinated with fixed concentrations of taurocholate (6 mM) and glycine (12 mM) added to various concentrations of 07C. The IC₅₀ value of 07C obtained from this regression is 7.60 μM. The germination assay kinetic graph that includes the 07C concentrations used to determine this IC₅₀ value can be found in Figure S3, Appendix.
2.3.4 Germination Profile of strain 05-1223-046

*C. difficile* strain 05-1223-046 displayed a strikingly different germination profile than the majority of tested strains. As expected, strain 05-1223-046 did not lose optical density when incubated with neat DMSO. Despite this, strain 05-1223-046 was also unable to germinate in the presence of 6 mM taurocholate with 12 mM glycine (Figure 2.14). After several trials resulting in the same pattern, we hypothesized that this strain may require a higher concentration of taurocholate and/or glycine for germination to occur. Regardless of increasing the concentration of one or both germinants, however, strain 05-1223-046 spores were still unable to germinate (Figure 2.14). Therefore, inhibition by the bile salt analog compounds *in vitro* could not be determined for this strain under these circumstances.
To rule out contamination, a C. difficile PCR confirmation was performed (Figure 2.15). Certain forward and reverse primers were chosen to support or disprove the presence various factors. One pair of primers was for the putative conjugative transposon protein Tn5397, which has target sites within C. difficile strain 630.\textsuperscript{181-183} Therefore, primers for that target sequence was used to detect strain 630. The absence of a band for Tn5397 established that the C. difficile strain present in the sample was not strain 630 (Figure 2.15, Lane 2). The Cs2gp primers were used to detect the presence of the C. sordelli reference strain Cs2.\textsuperscript{184} The absence of a band excluded C. sordelli as a contaminant in the sample (Figure 2.15, Lane 3). The primers for C. difficile toxin A (tcdA) was used to detect C. difficile and the universal primer pairs GM3 and GM4 were used to amply 16S rDNA found in bacteria.\textsuperscript{185} The appearance of bands for these sequences confirmed that the sample was C. difficile (Figure 2.15, Lane 4 and 5).
There could be a number possibilities as to why strain 05-1223-046 spores were unable to germinate with taurocholate and glycine. Perplexingly, in the Heeg et al. study, strain 05-1223-046 spores lose optical density by 74% when incubated with 0.1% taurocholate in BHIS.\textsuperscript{171} In that preparation, glycine was not added as a co-germinant. One hypothesis is that glycine may not be the most suitable co-germinant for spore germination of this particular strain. In a study by Figure 2.15. PCR confirmation of *C. difficile* strain 05-1223-046. PCR products from specific primers and genomic DNA were analyzed on 1% agarose gel. A 1 kb ladder is used for reference (Lane 1). The absence of a band for the putative conjugative transposon *tn5397* 672 confirms the absence of strain 630 (Lane 2). The absence of a band for *C. sordellii* reference strain Cs2 gp 927 verifies that *C. sordellii* contamination is not present in the sample (Lane 3). Bands for *fcd*A 1170 (Lane 4) and universal GM3/GM4 16S rDNA (Lane 5) confirm the presence of *C. difficile*. 
Howerton, Ramirez, and Abel-Santos, several other amino acid analogs were able to induce germination of strain 630 spores to different degrees.\textsuperscript{100} Individually, the amino acids were unable to trigger spore germination, but as a cocktail or in addition to taurocholate, spore were able to elicit germination.\textsuperscript{100} Testing different possible co-germinants in the germination of strain 05-1223-046 may be the next step in determining optimal germination conditions as well as discovering whether our proposed bile salt analog compounds can inhibit spore germination of that strain.

**2.3.5 Germination Profile of strain CDC 38**

*Clostridium difficile* strain CDC 38 spores were able to germinate in the presence of CamSA. Up to 100 μM CamSA, spores germinated at the same rate as the positive germination control containing taurocholate and glycine (Figure 2.16). Therefore, based on the cutoff criteria, CamSA is not considered to be an active germination inhibitor at concentrations below 100 μM.
Compound 07C greatly inhibited strain CDC 38 spore germination at low micromolar concentrations (Figure 2.16). At 1.5625 μM 07C, 79% of spores germinated. Only 43% of spores germinated at 6.25 μM. At 12.5 μM 07C, germination was inhibited by 77%. The IC<sub>50</sub> for 07C against the CDC 38 isolate was calculated to be 4.62 μM with an adjusted R<sup>2</sup> of 0.9897 (Figure 2.17).
2.3.6 Germination Profile of strain DH1834

Like the other tested isolates aside from strain 630, CamSA was unable to prevent spore germination of strain DH1834 (Figure 2.18). At 50 μM CamSA, spore germination resulted at a similar rate to the positive germination control. Even at 100 μM CamSA, comparable germination was exhibited. Thus, CamSA is not considered to be an active inhibitor against spores of this strain.
Germination inhibition was, however, evident with micromolar concentrations of 07C (Figure 2.18). At 1.5625 μM 07C, spore germination was down to 74%. At 50 μM 07C, loss of optical density was comparable to the negative germination control containing only DMSO and spores in germination buffer. Compound 07C’s IC$_{50}$ against strain DH1834 is 4.15 μM with an adjusted R$^2$ of 0.9720.
2.3.7 Germination Profile of strain 7004578

*Clostridium difficile* strain 7004578 sporulated poorly on BHIS agar. Hence, this strain was sporulated on *Clostridium difficile* sporulation media (SMC) agar containing 90 g Bacto peptone, 5 g proteose peptone, 1 g (NH$_4$)$_2$SO$_4$, 1.5 g Tris base, 15 g agar powder, and 5 g yeast per liter.$^{186-188}$ All other sporulation conditions remained the same.

Figure 2.19. IC$_{50}$ calculation for 07C against *C. difficile* strain DH1834 spores. The graph represents the dose-response curve of strain DH1834 spores germinated with fixed concentrations of taurocholate (6 mM) and glycine (12 mM) added to various concentrations of 07C. The IC$_{50}$ value of 07C obtained from this regression is 4.15 μM. The germination assay kinetic graph that includes the 07C concentrations used to determine this IC$_{50}$ value can be found in Figure S5, Appendix.
Oddly, spores of strain 7004578 were only able to germinate nominally in the presence of 6 mM taurocholate and 12 mM glycine. Spores, however, did not germinate in the negative germination control with DMSO. Like with strain 05-1223-046, we tested the effects of different concentrations of taurocholate and glycine on germination (Figure 2.20). Unlike with strain 05-1223-046, strain 7004578 spores germinated more when the concentrations of taurocholate and glycine were doubled. Loss of optical density, however, occurred to a much lesser degree than what was observed with other strains. Because optical density was only decreased by less than 10%, detecting slight changes in germination rates using multiple small concentrations of bile salt analog compounds would not be ideal for calculating IC\textsubscript{50}. Hence, the effects of CamSA and 07C on strain 7004578 spore germination could not be determined.
As with strain 05-1223-046, a PCR was run to confirm the presence of *C. difficile*. The same primers used for strain 05-1223-046's PCR confirmation was used for strain 7004578 (see section 2.3.3). No *C. sordellii* contamination was found in the strain 7004578 sample (Figure 2.21, Lane 3). Moreover, the sample was confirmed to be *C. difficile* based on the presence of bands for both *tcdA* and 16S rDNA (Figure 2.21, Lane 4 and 5).

**Figure 2.20.** Effects of taurocholate with glycine on the germination kinetic behavior of *C. difficile* strain 7004578 spores. Spores were resuspended in germination buffer and treated with neat DMSO (○), 6 mM taurocholate with 12 mM glycine (△), 6 mM taurocholate with 24 mM glycine (□), and 12 mM taurocholate with 24 mM glycine (◊). For clarity, data at five minute intervals are shown. The data points indicate the means from three independent measures (n = 3) and the error bars signify standard deviation from the mean.
Figure 2.21. PCR confirmation of *C. difficile* strain 7004578. PCR products from specific primers and genomic DNA were analyzed on 1% agarose gel. A 1 kb ladder is used for reference (Lane 1). The absence of a band for the putative conjugative transposon tn5397 672 confirms the absence of strain 630 (Lane 2). The absence of a band for *C. sordellii* reference strain Cs2 gp 927 verifies that *C. sordellii* contamination is not present in the sample (Lane 3). Bands for tcdA 1170 (Lane 4) and 16S rDNA (Lane 5) confirm the presence of *C. difficile*.

Since PCR confirmed that the sample was *C. difficile*, another possibility as to why the spores had trouble germinating was the SMC agar used to sporulation strain 7004578. To test this, a germination assay of strain 630 sporulated using SMC agar was performed and compared to a germination assay of strain 630 sporulated using BHIS agar. The assay revealed that spores of strain 630 that were sporulated on SMC agar were able to germinate normally when incubated with 6 mM taurocholate and 12 mM glycine. Although the relative optical density loss slightly differed between spores sporulated on BHIS agar versus SMC agar, the rate of germination remained the same. Therefore, it
is concluded that the SMC agar media was not responsible of strain 7004578's failure to germinate.

The choice of amino acid co-germinant could be a factor in the germination of strain 7004578 spores. Although doubling the concentrations of taurocholate and glycine did lower optical density slightly, the cause of this could be due to the doubling of the taurocholate itself since doubling of the glycine concentration alone had no difference in optical density loss compared to 6 mM taurocholate and 12 mM glycine data (Figure 2.20). If this were the case, taurocholate was still only able to induce germination minimally. A different amino acid co-germinant may be needed for optimal germination of this strain. This will be explored further in future studies.

2.3.8 Germination Profile of strain 8085054

For C. difficile strain 8085054 spores, CamSA was not an active inhibitor. Spore germination failed to be inhibited at 50 μM CamSA (Figure 2.22). Although CamSA may be active at much higher concentrations, it was not active with less than 100 μM.
Compound 07C appeared to be active against strain 8085054 at low micromolar concentrations (Figure 2.22). At just 1.5625 μM 07C, 54% of spore germination was inhibited. The IC\textsubscript{50} of 07C against strain 8085054 is 1.28 μM with an R\textsuperscript{2} of 0.9880 (Figure 2.23).

Figure 2.22. Effects of germinants and inhibitors on the germination kinetic behavior of C. difficile strain 8085054 spores. Spores were resuspended in germination buffer and treated with neat DMSO (○) or treated with a fixed concentration of taurocholate (6 mM) and glycine (12 mM) added to final concentrations of 0 μM of bile salt analog compound (Δ), 50 μM CamSA (●), 1.5625 μM 07C (▲), 6.25 μM 07C (♦), and 50 μM 07C (●). In actuality, more concentrations of bile salt analogs were used and data was collected every minute for 120 minutes (Figure S6, Appendix). For clarity, select concentrations are represented and data at five minute intervals are shown. The data points indicate the means from three independent measures (n = 3) and the error bars signify standard deviation from the mean.
2.3.9 Comparison of Germination Profiles Among All Tested C. difficile Strains

A total of eight C. difficile strains were tested for their responses to bile salt analog compounds CamSA and 07C. Although there are several similarities among the germination profiles of the strains, there are also some striking differences. These comparisons include differing responses to the germinants taurocholate and glycine and varying activity of bile salt analogs against each testable strain (Table 2.3).

Figure 2.23. IC<sub>50</sub> calculation for 07C against C. difficile strain 8085054 spores. The graph represents the dose-response curve of strain 8085054 spores germinated with fixed concentrations of taurocholate (6 mM) and glycine (12 mM) added to various concentrations of 07C. The IC<sub>50</sub> value of 07C obtained from this regression is 1.28 μM. The germination assay kinetic graph that includes the 07C concentrations used to determine this IC<sub>50</sub> value can be found in Figure S6, Appendix.
Table 2.3. Comparison of germination profiles among all tested *C. difficile* strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>CamSA IC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>07C IC&lt;sub&gt;50&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>630</td>
<td>58.3 μM*</td>
<td>8.19 μM</td>
</tr>
<tr>
<td>R20291</td>
<td>&gt; 100 μM</td>
<td>1.92 μM</td>
</tr>
<tr>
<td>9001966</td>
<td>&gt; 100 μM</td>
<td>7.60 μM</td>
</tr>
<tr>
<td>05-1223-046</td>
<td>TBD</td>
<td>TBD</td>
</tr>
<tr>
<td>CDC 38</td>
<td>&gt; 100 μM</td>
<td>4.62 μM</td>
</tr>
<tr>
<td>DH1834</td>
<td>&gt; 100 μM</td>
<td>4.15 μM</td>
</tr>
<tr>
<td>7004578</td>
<td>TBD</td>
<td>TBD</td>
</tr>
<tr>
<td>8085054</td>
<td>&gt; 100 μM</td>
<td>1.28 μM</td>
</tr>
</tbody>
</table>

TBD denotes IC<sub>50</sub> values that are yet to be determined for compounds where spores of those *C. difficile* strains were unable to germinate in the presence of 6 mM taurocholate and 12 mM glycine. Modifications will be made to determine potential co-germinants and conditions needed to promote germination in those strains.

*Value was obtained from Howerton, Ramirez, and Abel-Santos 2011.

All strains did not germinate in the neat DMSO negative germination control. Six of the eight strains were able to germinate with 6 mM taurocholate and 12 mM glycine as expected. These concentrations were chosen after previous research showed that they were optimal for germination of strain 630 and appropriate for germination inhibition studies. Those six strains observed loss of optical density by 30%-50% when incubated with taurocholate and...
glycine. Therefore, measuring IC$_{50}$ values for those strains was possible as long as germination rates corresponded to varying concentrations of compounds. However, strains 05-1223-046 and 7004578 spores failed to exhibit the same decrease in optical density as the other tested strains. After the possibilities of contamination and increasing concentrations were eliminated, it is hypothesized that other molecules may act as co-germinants of these two strains. Both strains are different PCR-ribotypes: 027 for strain 05-1223-046 and 078 for strain 7004578. Although no other 078 strains were tested, other 027 strains did not exhibit the same issue as 05-1223-046. Also, strain 7004578 spores were able to germinate slightly with doubled taurocholate and glycine concentrations, but strain 05-1223-046 spores were unable to display germinate distinct from the negative germination control. Future studies will include testing other amino acids as co-germins of these two strains. At the conclusion of this part of the in vitro germination study, the IC$_{50}$ for these two bile salt analog compounds against these strains have yet to be determined.

Of the remaining strains that were able to germinate with taurocholate and glycine, only strain 630 was capable of avoiding spore germination using less than 100 μM CamSA. A couple other strains were tested up to 1 mM final concentration of CamSA, but still did not display inhibition. CamSA may still be an inhibitor of spore germination, but may be weaker and require higher concentrations against the 6 mM taurocholate and 12 mM glycine. Spores from four strains (9001966, CDC 38, DH 1834, and 8085054) were tested for inhibition
using the naturally-occurring inhibitor chenodeoxycholate. Those four strains exhibited spore germination inhibition with 2 mM chenodeoxycholate. CamSA may be active against those other strains at concentrations above 1 mM, though this has not yet been tested.

While CamSA had an IC\textsubscript{50} of 58.3 µM against strain 630, 07C had IC\textsubscript{50} values of less than 10 µM against all testable strains. With strains R20291 and 8085054 spores, 07C’s IC\textsubscript{50} values were less than 2 µM. Compound 07C had IC\textsubscript{50} values between 4-5 µM against CDC 38 and DH 1834 isolates. The IC\textsubscript{50} of 07C against strains 630 and 9001966 were between 7-9 µM. With low IC\textsubscript{50} values, 07C may be a promising germination inhibitor in vivo, though other factors may also contribute to germination in an in vivo environment.

2.4 Conclusions

The diverse germination profiles of each C. difficile isolate reveal a glimpse of why CDI is such a fast-growing epidemic. While some strains respond to known germinants, others may require different molecules for germination to occur. Moreover, germination inhibition via bile salt analogs also vary among strains. Thus, finding a prophylactic solution for CDI will require a comprehensive study on C. difficile germination. Withal, the bile salt recognition site on C. difficile still has yet to be discovered.

In this study, some strains demonstrated typical behavior against known C. difficile germinants, taurocholate and glycine. However, spores of two strains,
05-1223-046 and 7004578, failed to germinate with those molecules. Spores of strain 630 were prevented from germinating in the presence of both CamSA and 07C; whereas, spores of all other testable strains were only inhibited by 07C.

Of the strains that could germinate in the presence of taurocholate and glycine, all observed inhibition of spore germination with the compound 07C with less than 100 μM. Furthermore, those six strains all had IC₅₀ values less than 10 μM. Although CamSA was only able to prevent germination with strain 630 spores with less than 100 μM, it may still be a potential inhibitor with other strains at higher concentrations that have not been tested at this time. These in vitro germination profiles may give insight into in vivo prophylactic treatment of CDI using the bile salt analogs against these different C. difficile strains. In addition, there may also be possible correlation between IC₅₀ values and CDI symptoms in vivo.
CHAPTER 3

IN VIVO STUDIES:

CDI SYMPTOMS IN THE ANIMAL MODEL

3.1 Introduction

Many animal models have been employed to study various aspects and mechanisms of CDI. Animals involved in CDI studies include, but are not limited to hamsters, mice, rabbits, guinea pigs, gnotobiotic piglets, Rhesus monkeys, and even zebrafish embryos. Furthermore, food animals like pigs and cattle and companion animals such as dogs and horses have been implicated in CDI studies, bringing into question foodborne- or animal interaction-related transmission of disease. The use of in vivo methods also provides insight into a plethora of host-pathogen interactions such as CDI pathophysiology and progression in hosts and C. difficile adaptation and colonization within the GI tract.

Animal C. difficile induction models aim to examine CDI symptoms in vivo. The most commonly and traditionally used animal CDI induction model is the hamster model. The hamster model was first used in antibiotic-associated colitis studies in the 1970s when several independent studies found links between antibiotic use, diarrhea, and colitis. C. difficile was later implicated as the culprit of the hemorrhagic lesions. After pre-treatment with antibiotics such as clindamycin, hamsters have been shown to be highly sensitive to CDI when given relatively low dosages of spores. Hamsters can elicit fulminant disease to
lethal within 48 hours. Although both humans and hamsters are susceptible to CDI after exposure to clindamycin, the disease progression in hamsters is much more extreme compared to in humans which can range from asymptomatic to severe symptoms. Also, unlike in humans where the site of infection is in the colon, hamsters CDI occurs in the cecum. Hamster models are also advantageous in studying colonization of C. difficile in the gut.

The mouse CDI model is a rodent model that has been developed in recent years. Mice are widely used as animal models and are less expensive than hamsters. In contrast to hamsters, mice are highly resistant to CDI and require a large inoculum of spores to establish significant infection symptoms. Similar to humans, mice symptoms can range from mild to severe disease signs. Clinical endpoint is slower in mice than in hamsters and lethality from CDI is less common in mice. Therefore, mice models are practical for studying spore shedding from the GI tract. Interestingly, mice that survive CDI often make a full recovery and do not relapse. A 2013 study by Howerton, Patra, and Abel-Santos showed that CDI-recovered mice did not show signs of relapse after being given another course of antibiotic treatment following a 14-day recovery period.

The Abel-Santos Laboratory has previously tested the bile salt analog CamSA in both the mouse and hamster CDI models. In the mouse CDI model, CamSA prevented CDI symptoms in female C57BL/6 mice with a single 50 mg/kg dose given immediately after challenge with C. difficile spores of strain.
630 and strain VPI 10463.\textsuperscript{157} Additionally, there was no observable toxicity at this dosage. Moreover, all mice that were treated with three doses of 50 mg/kg CamSA did not develop signs of CDI; whereas, mice treated with lower dosages of CamSA eventually developed some signs of CDI or succumbed to disease.\textsuperscript{157}

In the hamster CDI model, female Golden Syrian hamsters treated with 50 mg/kg CamSA showed a delayed onset of CDI symptoms and fatality compared to untreated hamsters. Treatment with vancomycin (which is commonly used to treat CDI) further delayed CDI symptoms and clinical endpoint in hamsters. In both cases, all hamsters eventually became moribund. However, the pairing of CamSA and vancomycin treatment in the hamster CDI model has shown synergistic effects in preventing CDI with an 80\% survival rate.

In this current study, the mouse CDI model was utilized to test whether CamSA and 07C can prevent CDI symptoms in mice challenged with spores of diverse \textit{C. difficile} strains. The results were also compared to the data obtained from the \textit{in vitro} germination profile analysis.

### 3.2 Materials and Methods

#### 3.2.1 Materials

\textit{C. difficile} strains R20291, 9001966, 05-1223-046, CDC 38, DH1834, 7004578, and 8085054 were generously donated by Professor Nigel Minton of the University of Nottingham. \textit{C. difficile} strain 630 was purchased from the ATCC. Synthesized bile salts were provided by Professor Steven M. Firestine of Wayne
State University A, or were previously synthesized in the Abel-Santos laboratory. Laboratory Rodent Diet was provided by the University of Nevada, Las Vegas animal care facility from LabDiet (St. Louis, MO, USA).

3.2.2 Animals

All procedures involving animals in this study were performed in accordance with the Guide for Care and Use of Laboratory Animals outlined by the National Institutes of Health. The protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Nevada, Las Vegas (Permit Number: R0914-297). Weaned female mice (strain C57BL/6) were purchased from Charles River Laboratories (Wilmington, MA, USA). Mice were housed in groups of five per cage at the University of Nevada, Las Vegas animal care facility. Upon arrival at the facility, mice were allowed to acclimate for one week prior to the start of experimentation. All bedding, cages, food, and water were autoclaved prior to housing animals. All post-challenge manipulations were performed within a biosafety level 2 laminar flow hood.

3.2.3 30-Day Compound Toxicity Regimen

Animals were given 300 mg/kg body weight (BW) of bile salt analog compound once per day for 30 days to test for possible toxicity. Bile salt analogs were administered via oral gavage with a total volume of 50 μL per dose. Neat
DMSO was used as a control in one cage of mice. Another cage of mice was given CamSA and mice in a third cage was given 07C. Fecal matter was collected per mice on days 0, 10, 30 for a separate study involving changes in gut microbiota. Weight changes were recorded on those days. Significant weight loss was defined as a loss of greater 15% original body weight. Mice were observed for signs of distress (i.e. lethargy and hunched posture) daily. At the end of the thirty-day trial, animals were sacrificed and necropsied to investigate for potential anatomical abnormalities.

3.2.4 Preparation of C. difficile Spores for Infection

C. difficile spores were harvested and purified using the Abel-Santos Laboratory Method as outlined in section 2.2.5 and section 3.2.3.1. Following spore purification, colony forming units (CFUs) were determined via plating methods. C. difficile spore inoculums were then optimized for infection in the murine CDI model.

3.2.4.1 C. difficile Spore Harvest and Purification

Prior to harvesting spores, inoculated plates were flooded with ice-cold deionized (DI) water. Cells and spores were then harvested by scraping bacteria colonies from the plates. The harvested cells and spores were pelleted via centrifugation at 8,000 × g for 5 minutes. The mixture was then resuspended in DI water and pelleted again. This washing step was repeated twice more. After the
three washing steps, the mixture was centrifuged through a 20% (5 mL) to 50% (10 mL) HistoDenz™ gradient at 18,200 × g for 30 minutes with no brake (Figure 2.1). The pelleted spore was then transferred to a clean centrifuge tube where it is washed five more times before being stored in DI water at 4°C. Spore purity was determined via Schaeffer-Fulton endospore staining method or phase contrast microscopy (see section 2.2.4). Spore preparations used were > 95% pure after centrifugation through the HistoDenz gradient (Figure 2.2).

**3.2.4.2 Determination of Colony Forming Units (CFUs)**

Spores were washed three times with DI water, heat shocked at 68°C for 30 minutes, then washed three more times. Heat-shocked spores were suspended into a fixed volume of DI water before being serially diluted in water. Serial dilutions were then plated on BHIS agar supplemented with 2% yeast extract, 0.1% L-cysteine-HCl, and 0.05% sodium taurocholate (prepared as described in section 2.2.2). Plates were incubated in an anaerobic chamber (10% CO₂, 10% H₂, 80% N₂) for 48 hours to yield individual colonies. Colonies were then counted to enumerate colony-forming units (CFUs).

**3.2.4.3 Optimization of C. difficile Spore Inoculum for Infection**

The murine CDI induction model used in this experiment was adapted from procedures published by Chen et al. in 2008 (Scheme 3.1). Mice were fed a standard laboratory rodent diet. Mice were given three consecutive days
of filtered antibiotic cocktail containing kanamycin (0.4 mg/ml), gentamycin (0.035 mg/ml), colistin (850 U/ml), metronidazole (0.215 mg/ml), and vancomycin (0.045 mg/ml). Mice were allowed to drink the antibiotic cocktail \textit{ad libitum}. Antibiotic cocktail was refreshed daily or as needed. Mice were then given autoclaved and filtered DI water for the remainder of the experiment. On the day prior to infection (24 hours before \textit{C. difficile} challenge), mice were given an intraperitoneal (IP) injection of 10 mg/kg BW clindamycin. On the day of infection, mice were challenged with $10^8$ \textit{C. difficile} spores via oral gavage. This heavy inoculum of spores ($10^8$ CFUs) has been shown to establish severe CDI with mice challenged with \textit{C. difficile} strain 630.$^{90}$ Mice were then observed for CDI symptoms for seven days post-challenge (discussed further in section 3.2.5).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Scheme_3.1.png}
\caption{Murine CDI induction model adapted from Chen \textit{et al.} 2008.$^{200}$ This model also represents the positive CDI control group regimen (spores only).}
\end{figure}

Scheme 3.1.
3.2.5 Animal CDI Model Regimens

The murine CDI prevention models used in this experiment were adapted and slightly modified from procedures published by Howerton, Patra, and Abel-Santos in 2013. Immediately following challenge, mice received 0 mg/kg BW or 50 mg/kg BW of one of two synthetic bile salt analogs via oral gavage: CamSA and 07C. One group of five antibiotic-treated mice was used as a negative CDI control group and were challenged with only DI water in place of C. difficile spores (Scheme 3.2). Positive CDI control mice received $10^8$ C. difficile spores and 0 mg/kg BW of the synthetic bile salt analog (Scheme 3.1). Experimental groups were challenged with $10^8$ C. difficile spores and received daily 50 mg/kg of the synthetic bile salt analog compound at 0, 24, and 48 hours post-challenge (Scheme 3.3) for a total of three doses.

![Diagram](image)

Scheme 3.2. Negative CDI control group regimen (no spores).
3.2.6 Observation of CDI Symptoms in Animals

Mice were observed for signs of CDI twice daily and were scored according a CDI scoring rubric (Table 3.1). This rubric has been adapted and slightly modified from a previously published rubric by Howerton, Patra, and Abel-Santos in 2013. CDI signs included anogenital redness, lethargic/distressed behavior, presence of diarrhea or soiled bedding, wet tail, hunched posture, and weight loss.
Scores from each animal were tallied to determined severity of CDI symptoms (Table 3.2). Animals scoring equal to or less than 2 were undistinguishable from noninfected controls and were considered non-diseased. Animals scoring 3–4 were considered to have mild CDI. Animals scoring 5–6 were considered to have moderate CDI. Animals scoring greater than 6 were considered to have severe CDI and were immediately sacrificed.

Table 3.1. CDI Scoring Rubric for mouse CDI model.

<table>
<thead>
<tr>
<th>CDI Disease Signs</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pink anogenital area</td>
<td>1</td>
</tr>
<tr>
<td>Red anogenital area</td>
<td>2</td>
</tr>
<tr>
<td>Lethargy/distress</td>
<td>2</td>
</tr>
<tr>
<td>Increased diarrhea/soiled bedding</td>
<td>2</td>
</tr>
<tr>
<td>Mild wet tail</td>
<td>1</td>
</tr>
<tr>
<td>Wet tail</td>
<td>2</td>
</tr>
<tr>
<td>Hunched posture</td>
<td>2</td>
</tr>
<tr>
<td>8-15% weight loss</td>
<td>1</td>
</tr>
<tr>
<td>&gt; 15% weight loss</td>
<td>2</td>
</tr>
</tbody>
</table>

Adapted and modified from Howerton, Patra, and Abel-Santos 2013.\textsuperscript{157}

Table 3.2. Mouse CDI symptoms severity with corresponding scores.

<table>
<thead>
<tr>
<th>CDI Symptoms Severity</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-diseased</td>
<td>≤ 2</td>
</tr>
<tr>
<td>Mild CDI</td>
<td>3-4</td>
</tr>
<tr>
<td>Moderate CDI</td>
<td>5-6</td>
</tr>
<tr>
<td>Severe CDI (euthanized)</td>
<td>&lt; 6</td>
</tr>
</tbody>
</table>
3.2.7 Statistical Analysis

Severity of symptoms were analyzed via box-and-whisker plots. Data from the plots were expressed as mean ± standard deviation. Standard deviations represent a minimum of three independent values (n ≥ 3, where n is one mouse). A one-tailed unpaired Student’s T-test was performed to determine statistically significant difference between two means (positive CDI control group versus experimental group). Statistical significance was determined as P values of < 0.1 (*), < 0.05 (**), or < 0.01 (***)

3.3 Results and Discussion

3.3.1 Observable Toxicity of Bile Salt Analogs in Mice

Of the bile acids present in the gut, approximately 95% of them get recycled back into enterohepatic circulation. Some bile salts, such as chenodeoxycholate, are very readily absorbed by the intestines and brought back to the liver, while others, such as lithocholate (formed from unabsorbed chenodeoxycholate), are poorly absorbed and remain in the gut. Although most of the remaining 5% of bile acids are eliminated through excretion, accumulation of too much of bile acid like lithocholate can lead to toxic effects. Thus, the two synthetic bile salt analog compounds, CamSA and 07C, were tested for any toxic effects to mice. Mice were given 300 mg/kg of either CamSA or 07C every day for thirty days and were observed for signs of toxicity.
Neat DMSO was used as a control since bile salt analogs were dissolved in DMSO.

Body weight loss is often used as a measure of toxicity in pharmacodynamic studies.\textsuperscript{199,200,202} Weight loss in mice was calculated as percent change from the weight on day 0. Mice that received neat DMSO, 300 mg/kg CamSA, and 300 mg/kg 07C all did not experience weight loss greater than 10\% over the course of the trial (Figure 3.1). Moreover, mice did not appear to display signs of distress such as lethargy or having a hunched posture. Mice activity was monitored for 15-20 minutes after oral gavage every day. Mice appeared to exhibit normal behavior following oral gavage.
Furthermore, necropsy was performed to check for anatomical anomalies that may be caused by the compounds given. Of all fifteen mice tested, only one mouse in the 300 mg/kg CamSA cage succumbed to death on day 21. After necropsy of the animal, an air pocket was found in the stomach. This is most likely due to an oral gavage error when air may have accidentally been

Figure 3.1. Mean relative percent weight change of mice in bile salt analog toxicity study. Data points represent mean relative percent weight change compared to day 0 from five independent measures (n =5). Error bars indicate standard deviation from the mean. A) Average weight of mice that received neat DMSO daily for thirty days (solid line). B) Average weight of mice that received 300 mg/kg CamSA daily for thirty days (dotted line). C) Average weight of mice that received 300 mg/kg 07C daily for thirty days (dashed line).
introduced. No organomegaly or other abnormalities of any other organs were found, making the cause of death unlikely to be due to compound toxicity.

All other mice survived the entirety of the trial. Upon necropsy, all organs were intact and no enlargement was discovered. Organ enlargements of concern included digestive organs such as the liver, pancreas, spleen stomach, small intestine, and colon. The liver was of interest as it is responsible for recycling bile salts. Attention was also brought to the intestines as they are the sight of reabsorption of bile salts. Neither had visibly observable damage or enlargement. With weight loss, signs of distress, and necropsy surveillance in consideration, neat DMSO, CamSA, and 07C all appeared to have no observable toxicity to mice.

3.3.2 Analysis of CDI Symptoms in Mice Challenged with DI Water

One cage of five mice challenged with DI water in place of C. difficile spores was used as a negative CDI control. These mice did not receive any bile salt analog compound treatment. Other than incidental weight loss that was less than 15% original body weight prior to challenge, the mice did not develop any signs of CDI. Thus, the mice received a maximum score of a 1 indicating no disease (Figure 3.2).
3.3.3 Analysis of CDI Symptoms in Mice Challenged with strain 630

CamSA and 07C both prevented CDI in mice challenged with *C. difficile* strain 630. Mice that were challenged with $10^8$ strain 630 spores (positive CDI control), developed CDI symptoms, though to varying degrees (Figure 3.3A). At 48 hours post-infection, the mean symptoms severity for the positive control cage was 4.2, indicating mild-to-moderate CDI (Table 3.3). Although one animal received a score of a 3 (mild CDI), another succumbed to disease with a score of an 8 (severe CDI). Positive control mice eventually recovered after 72 hours and were monitored for at least seven more days. No surviving mice exhibited relapsed CDI.
Figure 3.3. CDI symptoms severity in mice challenged with strain 630 spores. Data points represent each animal’s symptoms severity based on the CDI scoring rubric. Overlap of data points occurs when animals share the same score. Error bars indicate standard deviation from the mean. Standard deviations represent a minimum of three independent values (n ≥ 3). Experimental groups were statistically significant (unpaired Student’s T-test) compared to the positive control group at p < 0.1 (*), p < 0.05 (**), and p < 0.01 (**). A) Symptoms severity of untreated positive control mice (○). B) Symptoms severity of mice treated with three doses of 50 mg/kg CamSA (□). C) Symptoms severity of mice treated with three doses of 50 mg/kg 07C (△).
Table 3.3. Mean CDI symptoms severity for mice challenged with strain 630 spores.

<table>
<thead>
<tr>
<th>630 Group</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
<th>96 h</th>
<th>120 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.0</td>
<td>4.2</td>
<td>0.5</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>CamSA-treated</td>
<td>0</td>
<td>0.2</td>
<td>0.2</td>
<td>0</td>
<td>0.2</td>
</tr>
<tr>
<td>07C-treated</td>
<td>0.4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

KEY: light green = non-disease, yellow-orange = mild-to-moderate CDI

Compared to the mild-to-moderate CDI found in the untreated mice, CamSA- and 07C-treated mice were mostly asymptomatic. Scores of 1 by individual mice were caused by slight changes in weight which can sometimes be an indicator of progressive CDI when weight continues to decrease over a period of time. CamSA-treated mice had a maximum mean score of 0.2, signifying no disease (Figure 3.3B, Table 3.3). Likewise, 07C-treated mice had a maximum mean score of 0.4, also indicative of no CDI (Figure 3.3C, Table 3.3). Thus, both compounds were effective at preventing CDI symptoms in strain 630-infected mice at the three 50 mg/kg doses given.

3.3.4 Analysis of CDI Symptoms in Mice Challenged with strain R20291

Mice challenged with C. difficile strain R20291 spores exhibited slightly higher CDI scores than strain 630. Untreated positive CDI control mice had a maximum mean symptoms severity of 5.2 at 48 hours post-infection, indicating moderate CDI (Figure 3.4A, Table 3.4). At the lower end, two mice had mild CDI with scores of 4. At the higher end, one mouse became moribund with severe
CDI, receiving a score of 7. The remaining mice received scores of 5 and 6 (moderated CDI).

Figure 3.4. CDI symptoms severity in mice challenged with strain R20291 spores. Data points represent each animal’s symptoms severity based on the CDI scoring rubric. Overlap of data points occurs when animals share the same score. Error bars indicate standard deviation from the mean. Standard deviations represent a minimum of three independent values (n ≥ 3). Experimental groups were statistically significant (unpaired Student’s T-test) compared to the positive control group at p < 0.1 (*), p < 0.05 (**), and p < 0.01 (**). A) Symptoms severity of untreated positive control mice (○). B) Symptoms severity of mice treated with three doses of 50 mg/kg CamSA (□). C) Symptoms severity of mice treated with three doses of 50 mg/kg 07C (△).
CamSA-treated mice were not protected from CDI. At 48 hours, CDI symptoms in CamSA-treated mice were not significantly different from the positive control (Figure 3.4B). Although the mean symptoms severity is 4.2 (mild-to-moderate CDI) for CamSA-treated mice compared to 5.2 (moderate CDI) in the positive control, the spread is much larger in the CamSA-treated mice (SD = 2.58 vs. 1.30 in positive control mice) (Table 3.4). This may be due to differences in germination rates in vivo as well as a number of other possible factors. Since the in vitro germination assay reveal that CamSA was not an inhibitor of germination strain 630 spores with less than 100 μM of compound, it is not surprising that CamSA was unable to prevent CDI against strain 630 spores. It is possible that CamSA does not inhibit strain 630 spore germination even at higher concentrations.

Compound 07C reduced and delayed CDI symptoms in strain 630-challenged mice (Figure 3.4C). 07C-treated mice still developed CDI symptoms, but only with a maximum mean symptoms severity score of 2.6, which reaches

### Table 3.4. Mean CDI symptoms severity for mice challenged with strain R20291 spores.

<table>
<thead>
<tr>
<th>R20291 Group</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
<th>96 h</th>
<th>120 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.0</td>
<td>5.2</td>
<td>2.0</td>
<td>1.25</td>
<td>0.75</td>
</tr>
<tr>
<td>CamSA-treated</td>
<td>1.0</td>
<td>4.2</td>
<td>4.75</td>
<td>1.25</td>
<td>0</td>
</tr>
<tr>
<td>07C-treated</td>
<td>0</td>
<td>2.0</td>
<td>2.6</td>
<td>0.2</td>
<td>0.6</td>
</tr>
</tbody>
</table>

**KEY:** light green = non-disease, green = non-diseased-to-mild CDI, yellow-orange = mild-to-moderate CDI, orange = moderate CDI.
into the mild CDI range (Table 3.4). One mouse approached a score of a 4, indicating mild CDI. While the positive control mice displayed maximum symptoms at 48 hours post-infection, 07C-treated mice CDI peaked at 72 hours post-challenge. Considering that mice were given the last dose of 50 mg/kg 07C at 48 hours post-infection, it is possible that the discontinuation of treatment resulted in the delay of CDI. One study revealed that strain 630 spores shed over a 96-hour timeframe.\textsuperscript{80} It is unknown when strain R20291 spores fully shed from feces, though they may remain in the gut over the 48 hours (after treatment is discontinued). Therefore, extending bile salt analog treatment may fully prevent germination of strain R20291 spores in the gut until they are shed.

All surviving mice were kept for at least seven more days for observation. None of the mice had disease relapse. All surviving mice appeared to fully recover from initial CDI.

### 3.3.5 Analysis of CDI Symptoms in Mice Challenged with strain 9001966

*C. difficile* strain 9001966-challenged mice begin to have mild-to-moderate CDI starting at 24 hours post-challenge (Figure 3.5A). At 48 hours post-challenge, two mice reached scores of 6 (moderate CDI) (Table 3.5). Both at 24 and 48 hours post-challenge, mean symptoms severity was 4.6 (mild-to-moderate CDI). At 72 hours, maximum mean symptoms severity was reached (score of 5.2) and all mice had moderate CDI. Interestingly, the mice remain sick until 120 hours post-challenge, when they begin to recover. Although data past
120 hours is not shown, the mice continued to be monitored and all fully recovered from CDI by 144 hours post-challenge.

Figure 3.5. CDI symptoms severity in mice challenged with strain 9001966 spores. Data points represent each animal’s symptoms severity based on the CDI scoring rubric. Overlap of data points occurs when animals share the same score. Error bars indicate standard deviation from the mean. Standard deviations represent a minimum of three independent values (n ≥ 3). Experimental groups were statistically significant (unpaired Student’s T-test) compared to the positive control group at p < 0.1 (*), p < 0.05 (**), and p < 0.01 (**). A) Symptoms severity of untreated positive control mice (○). B) Symptoms severity of mice treated with three doses of 50 mg/kg CamSA (□). C) Symptoms severity of mice treated with three doses of 50 mg/kg 07C (△).
Table 3.5. Mean CDI symptoms severity for mice challenged with strain 9001966 spores.

<table>
<thead>
<tr>
<th>9001966 Group</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
<th>96 h</th>
<th>120 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.6</td>
<td>4.6</td>
<td>5.2</td>
<td>4.6</td>
<td>0.4</td>
</tr>
<tr>
<td>CamSA-treated</td>
<td>0.5</td>
<td>1.25</td>
<td>3.5</td>
<td>2.0</td>
<td>0.75</td>
</tr>
<tr>
<td>07C-treated</td>
<td>0.25</td>
<td>1.0</td>
<td>0.75</td>
<td>3.0</td>
<td>2.25</td>
</tr>
</tbody>
</table>

**KEY:** light green = non-disease, green = non-diseased-to-mild CDI, yellow = mild CDI, yellow-orange = mild-to-moderate CDI, orange = moderate CDI

Although CamSA and 07C did not fully protect CDI in strain 9001966-challenged mice, both compounds reduced CDI symptoms significantly (Figure 3.5B-C). Average symptoms severity scores were 3.5 for CamSA-treated mice and 3.0 for 07C treated mice, both representing mild CDI (Table 3.5). Curiously, both onsets of maximum symptoms also appeared later than the control. This, again, could be related to the cessation of treatment, allowing remaining spores in the gut to germinate.

CamSA was unable to prevent strain 9001966 spore germination in vitro, though it was able to reduce CDI symptoms in mice. CamSA may be active against strain 9001966 spores in vitro at higher concentrations. 07C was able to inhibit germination of strain 9001966 spores with an IC$_{50}$ of 7.60 μM. Oddly, although the IC$_{50}$ of 07C against strain 630 is slightly higher at 8.19 μM, 07C fully prevented CDI in strain 630-challenged mice. Therefore, there may not be a direct correlation in in vitro IC$_{50}$ values and in vivo CDI symptoms severity. This
may be due to *C. difficile* inter-strain variability or other variables present in each individual mouse’s gut.

3.3.6 Analysis of CDI Symptoms in Mice Challenged with strain 05-1223-046

*In vivo*, both CamSA and 07C were able to reduce CDI symptoms in mice given *C. difficile* strain 05-1223-046 spores (Figure 3.6B-C). Mean CDI symptoms severity scores at all time points indicate non-diseased mice. However, untreated mice did not develop as intense symptoms as untreated mice given other strains (Figure 3.6A). The difference between treated and untreated mice was still statistically significant, though to different degrees depending on the times point. Maximum mean symptoms severity in untreated mice occurs at 24 and 48 hours and is only a score of 3.0, which is at the lower end of mild CDI (Table 3.6). This brings speculation about strain 05-1223-046’s resistance to germination with the natural germinants taurocholate and glycine.
Figure 3.6. CDI symptoms severity in mice challenged with strain 05-1223-046 spores. Data points represent each animal's symptoms severity based on the CDI scoring rubric. Overlap of data points occurs when animals share the same score. Error bars indicate standard deviation from the mean. Standard deviations represent a minimum of three independent values ($n \geq 3$). Experimental groups were statistically significant (unpaired Student’s T-test) compared to the positive control group at $p < 0.1$ (*), $p < 0.05$ (**), and $p < 0.01$ (**). A) Symptoms severity of untreated positive control mice (○). B) Symptoms severity of mice treated with three doses of 50 mg/kg CamSA (□). C) Symptoms severity of mice treated with three doses of 50 mg/kg 07C (∆).
3.3.7 Analysis of CDI Symptoms in Mice Challenged with strain CDC 38

Mice challenged with *C. difficile* strain CDC 38 spores displayed onset of maximum mean symptoms severity score of 4.2 at 24 hours post-infection, signifying mild-to-moderate CDI (Figure 3.7A, Table 3.7). The intensity of CDI steady decreased over time until all mice eventually recovered around 120 hours post-infection.

CamSA was unable to prevent or significantly reduce CDI symptoms in strain CDC 38-infected mice (Figure 3.7B). The highest achieved scored was by one mouse at 24 hours with a score of 5 (moderate CDI). The remaining mice reached mild-to-moderate CDI at 48 hours. At that time, the maximum mean symptoms severity score of 3.8 (mild CDI) was attained (Table 3.7).
Figure 3.7. CDI symptoms severity in mice challenged with strain CDC 38 spores. Data points represent each animal’s symptoms severity based on the CDI scoring rubric. Overlap of data points occurs when animals share the same score. Error bars indicate standard deviation from the mean. Standard deviations represent a minimum of three independent values (n ≥ 3). Experimental groups were statistically significant (unpaired Student’s T-test) compared to the positive control group at p < 0.1 (*), p < 0.05 (**), and p < 0.01 (**). A) Symptoms severity of untreated positive control mice (○). B) Symptoms severity of mice treated with three doses of 50 mg/kg CamSA (□). C) Symptoms severity of mice treated with three doses of 50 mg/kg 07C (△).
Table 3.7. Mean CDI symptoms severity for mice challenged with strain CDC 38 spores.

<table>
<thead>
<tr>
<th>CDC 38 Group</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
<th>96 h</th>
<th>120 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.2</td>
<td>3.4</td>
<td>2.8</td>
<td>2.0</td>
<td>0.4</td>
</tr>
<tr>
<td>CamSA-treated</td>
<td>0</td>
<td>3.2</td>
<td>3.8</td>
<td>1.6</td>
<td>1.0</td>
</tr>
<tr>
<td>07C-treated</td>
<td>0.4</td>
<td>3.4</td>
<td>3.2</td>
<td>1.8</td>
<td>1.6</td>
</tr>
</tbody>
</table>

**KEY:** light green = non-disease, green = non-diseased-to-mild CDI, yellow = mild CDI, yellow-orange = mild-to-moderate CDI

Of all the strains tested in the mouse CDI model, 07C appears to be the least potent against strain CDC 38. 07C-treated mice still developed mild CDI with a maximum mean symptoms severity of 3.4 (mild CDI) at 48 hours post-challenge (Figure 3.7C). However, this was a mild score reduction from the untreated mice mean score of 4.2 (mild-to-moderate) at 24 hours post-challenge (Table 3.7). This decrease is statistically insignificant. Ironically, 07C had quite a low IC50 of 4.62 μM against strain CDC 38. *In vivo*, mice may require higher dosages of 07C for it to be an effective prophylactic against this strain.

### 3.3.8 Analysis of CDI Symptoms in Mice Challenged with strain DH1834

*C. difficile* strain DH1834-challenged mice had a steady progression to maximum symptoms (Figure 3.8A). Starting at 24 hours, mice began to develop CDI symptoms. However, maximum mean symptoms severity was occurred at 72 hours, with a score of 4.6, indicating mild-to-moderate CDI (Table 3.8). One mouse reached a score of 7 and reached clinical endpoint. At 96 hours,
remaining mice recovered and remained asymptomatic for at least seven more days.

Figure 3.8. CDI symptoms severity in mice challenged with strain DH1834 spores. Data points represent each animal’s symptoms severity based on the CDI scoring rubric. Overlap of data points occurs when animals share the same score. Error bars indicate standard deviation from the mean. Standard deviations represent a minimum of three independent values (n ≥ 3). Experimental groups were statistically significant (unpaired Student’s T-test) compared to the positive control group at p < 0.1 (*), p < 0.05 (**), and p < 0.01 (***). A) Symptoms severity of untreated positive control mice (○). B) Symptoms severity of mice treated with three doses of 50 mg/kg CamSA (□). C) Symptoms severity of mice treated with three doses of 50 mg/kg 07C (△).
CamSA did not prevent CDI in mice challenged with strain DH1834 spores (Figure 3.8B). Mice developed maximum mean symptoms severity score of 4.6 (mild-to-moderate CDI) at 24 hours (Table 3.8). Symptoms severity continued to drop from 48 hours on. 07C also did not reduce CDI symptoms to a significant degree, however, it did significantly cut symptoms severity in the following days (Figure 3.8B). Maximum mean symptoms severity in 07C-treated mice was 3.4, which is a slight decrease from the positive CDI control (Table 3.8). As 07C also has a low IC\textsubscript{50} of 4.15 μM against strain DH1834 spores, other in vivo factors may play a role in the germination of strain DH1834 spores.

### 3.3.9 Analysis of CDI Symptoms in Mice Challenged with strain 7004578

Like with strain 05-1223-046, the maximum mean symptoms severity for strain 7004578 was also in the mild CDI range (Figure 3.9A). In the in vitro germination profile study, both of these strains had difficulty germinating in the presence of the natural activators. However, the reason for the low mean score of 3.2 at 24
hours post-challenge was mainly due to two animals remaining asymptomatic throughout the duration of the experiment (Table 3.9). Of the three remaining mice, two mice reached clinical endpoint: one at 24 hours and the other at 48 hours. The third mouse kept a steady score of 4 (mild CDI) from 24 hours to 72 hours, until it began to recover at 96 hours. Although the two non-diseased mice could not be considered as outliers, another graph was added to the figure below to provide an alternative view of disease progression (Figure 3.9B).
Figure 3.9. CDI symptoms severity in mice challenged with strain 7004578 spores. Data points represent each animal’s symptoms severity based on the CDI scoring rubric. Overlap of data points occurs when animals share the same score. Error bars indicate standard deviation from the mean. Standard deviations represent a minimum of three independent values (n ≥ 3). Experimental groups were statistically significant (unpaired Student’s T-test) compared to the positive control group at p < 0.1 (*), p < 0.05 (**), and p < 0.01 (**). A) Symptoms severity of untreated positive control mice (○). B) Symptoms severity of positive control mice with data points for two asymptomatic mice eliminated (◊). C) Symptoms severity of mice treated with three doses of 50 mg/kg CamSA (□). D) Symptoms severity of mice treated with three doses of 50 mg/kg 07C (∆).
CamSA was not effective at preventing or reducing CDI symptoms of mice given strain 7004578 spores. There is no statistically significant difference between symptoms of CamSA-treated mice and untreated mice (Figure 3.9C). Maximum mean symptoms severity for CamSA-treated mice occurred at 48 hours with a score of 3.25 (mild CDI) (Table 3.9). However, 07C was able to prevent CDI altogether with strain 7004578-challenged mice remaining non-disease throughout duration of the experiment (Figure 3.9D).

### 3.3.10 Analysis of CDI Symptoms in Mice Challenged with strain 8085054

CDI symptoms of C. difficile strain 8085054-infected mice displayed a similar progression of disease to mice infected with strain 630 and strain R20291 (Figure 3.10A). Positive CDI control mice that received strain 8085054 spores presented with mild CDI (mean score of 3.4) at 48 hours post-infection (Table 3.10). At 72 hours and beyond, mice gradually recovered and did not relapse.
Figure 3.10. CDI symptoms severity in mice challenged with strain 8085054 spores. Data points represent each animal’s symptoms severity based on the CDI scoring rubric. Overlap of data points occurs when animals share the same score. Error bars indicate standard deviation from the mean. Standard deviations represent a minimum of three independent values (n ≥ 3). Experimental groups were statistically significant (unpaired Student’s T-test) compared to the positive control group at p < 0.1 (*), p < 0.05 (**), and p < 0.01 (**). A) Symptoms severity of untreated positive control mice (○). B) Symptoms severity of positive control mice with data points for two asymptomatic mice eliminated (◊). C) Symptoms severity of mice treated with three doses of 50 mg/kg CamSA (□). D) Symptoms severity of mice treated with three doses of 50 mg/kg 07C (△).
Both CamSA and 07C prevented or reduced CDI symptoms for this strain (Figure 3.10B-C). CamSA also appeared maximum symptoms in a couple mice until 96 hours. Most mice were non-diseased throughout the experiment (Table 3.10). 07C-treated mice received scores of 0 to 2, marking them as non-diseased (Table 3.10). In vitro, 07C had the lowest IC\(_{50}\) value (1.28 μM) against strain 8085054. In this case, the in vitro data reflects the in vivo results.

### 3.3.11 Comparison of CDI Symptoms Among All Tested C. difficile Strains

The eight tested C. difficile strains exhibited distinct CDI disease progression and symptoms severity (Table 3.11). The range in onset of maximum symptoms also vary among strains. Strains R20291 and 9001966 both presented with the highest maximum mean symptoms severity with a score of 5.2, indicating moderate CDI. Some of those mice also became moribund. Strain R20291 is the hypervirulent strain of ribotype 027. Therefore, it is expected that mice given this strain would display more severe CDI. Strains 630, CDC 38, and DH1834 all showing mild-to-moderate CDI. However, the onset of maximum

<table>
<thead>
<tr>
<th>8085054 Group</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
<th>96 h</th>
<th>120 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.4</td>
<td>3.4</td>
<td>2.4</td>
<td>1.2</td>
<td>0.4</td>
</tr>
<tr>
<td>CamSA-treated</td>
<td>0</td>
<td>0</td>
<td>0.2</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>07C-treated</td>
<td>0</td>
<td>0.6</td>
<td>1.2</td>
<td>0</td>
<td>0.8</td>
</tr>
</tbody>
</table>

**KEY:** light green = non-disease, green = non-diseased-to-mild CDI, yellow = mild CDI

Table 3.10. Mean CDI symptoms severity for mice challenged with strain 8085054 spores.
symptoms all vary between the three strains. Strain 05-1223-046, 7004578, and 8085054 only presented with averages of mild CDI, although some mice became more symptomatic than others.

Table 3.11. Comparison of mean CDI symptoms for all positive control mice.

<table>
<thead>
<tr>
<th>Strain</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
<th>96 h</th>
<th>120 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>630</td>
<td>2.0</td>
<td>4.2</td>
<td>0.5</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>R20291</td>
<td>1.0</td>
<td>5.2</td>
<td>2.0</td>
<td>1.25</td>
<td>0.75</td>
</tr>
<tr>
<td>9001966</td>
<td>4.6</td>
<td>4.6</td>
<td>5.2</td>
<td>4.6</td>
<td>0.4</td>
</tr>
<tr>
<td>05-1223-046</td>
<td>3.0</td>
<td>3.0</td>
<td>2.4</td>
<td>2.0</td>
<td>0</td>
</tr>
<tr>
<td>CDC 38</td>
<td>4.2</td>
<td>3.4</td>
<td>2.8</td>
<td>2.0</td>
<td>0.4</td>
</tr>
<tr>
<td>DH1834</td>
<td>4.0</td>
<td>3.4</td>
<td>4.6</td>
<td>2.75</td>
<td>2.5</td>
</tr>
<tr>
<td>7004578</td>
<td>3.2</td>
<td>2.75</td>
<td>1.33</td>
<td>0.67</td>
<td>0.33</td>
</tr>
<tr>
<td>8005054</td>
<td>0.4</td>
<td>3.4</td>
<td>2.4</td>
<td>1.2</td>
<td>0.4</td>
</tr>
</tbody>
</table>

KEY: light green = non-disease, green = non-diseased-to-mild CDI, yellow = mild CDI, yellow-orange = mild-to-moderate CDI, orange = moderate CDI

Mice also responded differently to bile salt analog treatment depending on the C. difficile strain used for challenge (Table 3.11 and 3.12). CamSA was not expected to be able to prevent CDI any C. difficile strains other than strain 630 since the in vitro data suggested that it was only a potent inhibitor against that strain. However, CamSA showed some promising results with some strains in the mouse CDI model. CamSA prevented CDI in strain 630, as predicted, but also in strains 05-1223-046 and 8085054. Symptoms reduction was seen in strain 9001966.
in CamSA-treated mice. Thus, the concentration of CamSA may need to be increased much more against these strains in vitro to possibly determine inhibitory concentrations against germination. In contrast, CamSA was not effective against strains R20291, CDC 38, DH1834, and 7004578.

Table 3.12. Comparison of mean CDI symptoms for all CamSA-treated mice.

<table>
<thead>
<tr>
<th>Strain</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
<th>96 h</th>
<th>120 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>630</td>
<td>0</td>
<td>0.2</td>
<td>0.2</td>
<td>0</td>
<td>0.2</td>
</tr>
<tr>
<td>R20291</td>
<td>1.0</td>
<td>4.2</td>
<td>4.75</td>
<td>1.25</td>
<td>0</td>
</tr>
<tr>
<td>9001966</td>
<td>0.5</td>
<td>1.25</td>
<td>3.5</td>
<td>2.0</td>
<td>0.75</td>
</tr>
<tr>
<td>05-1223-046</td>
<td>0</td>
<td>0.2</td>
<td>1.2</td>
<td>0.4</td>
<td>0</td>
</tr>
<tr>
<td>CDC 38</td>
<td>0</td>
<td>3.2</td>
<td>3.8</td>
<td>1.6</td>
<td>1.0</td>
</tr>
<tr>
<td>DH1834</td>
<td>4.6</td>
<td>2.75</td>
<td>3.0</td>
<td>0.75</td>
<td>1.5</td>
</tr>
<tr>
<td>7004578</td>
<td>2.75</td>
<td>3.25</td>
<td>2.0</td>
<td>1.5</td>
<td>1.75</td>
</tr>
<tr>
<td>8005054</td>
<td>0</td>
<td>0</td>
<td>0.2</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

**KEY:** light green = non-disease, green = non-diseased-to-mild CDI, yellow = mild CDI, yellow-orange = mild-to-moderate CDI, orange = moderate CDI

07C significantly prevented, reduced, or reduced and delayed CDI symptoms in mice challenged with almost all strains tested. The only strain that showed the most insignificant symptom reduction against was strain CDC 38. In strains 630, 05-1223-046, 7004578, and 8085054, mice were considered non-diseased when treated with 07C. Symptom onset was delayed with the hypervirulent strain R20291. However, CDI was greatly reduced in mice.
challenged with strain R20291. CDI symptoms were reduced and delayed with strain 9001966-infected mice. Finally, with strain DH1834, maximum symptoms were only mildly reduced, but mice did exhibit delayed protection at the subsequent time points.

Table 3.13. Comparison of mean CDI symptoms for all 07C-treated mice.

<table>
<thead>
<tr>
<th>Strain</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
<th>96 h</th>
<th>120 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>630</td>
<td>0.4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>R20291</td>
<td>0</td>
<td>2.0</td>
<td>2.6</td>
<td>0.2</td>
<td>0.6</td>
</tr>
<tr>
<td>9001966</td>
<td>0.25</td>
<td>1.0</td>
<td>0.75</td>
<td>3.0</td>
<td>2.25</td>
</tr>
<tr>
<td>05-1223-046</td>
<td>0.6</td>
<td>1.0</td>
<td>1.4</td>
<td>1.8</td>
<td>0.8</td>
</tr>
<tr>
<td>CDC 38</td>
<td>0.4</td>
<td>3.4</td>
<td>3.2</td>
<td>1.8</td>
<td>1.6</td>
</tr>
<tr>
<td>DH1834</td>
<td>3.4</td>
<td>0.8</td>
<td>0.8</td>
<td>0.2</td>
<td>0.6</td>
</tr>
<tr>
<td>7004578</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.4</td>
<td>0.2</td>
</tr>
<tr>
<td>8085054</td>
<td>0</td>
<td>0.6</td>
<td>1.2</td>
<td>0</td>
<td>0.8</td>
</tr>
</tbody>
</table>

KEY: light green = non-disease, green = non-diseased-to-mild CDI, yellow = mild CDI, yellow-orange = mild-to-moderate CDI, orange = moderate CDI

3.4 Conclusions

The mouse CDI model has demonstrated the powerful potential of bile salt analogs in the prophylactic treatment of CDI in mice. By using the in vitro germination profile data, we made predictions about the outcomes of the in vivo mouse CDI model. However, although some in vitro data reflected in vivo findings, others did not show a direct correlation. This may be due to different
germination rates of spores of certain C. difficile strains in vivo. This could be caused by, but not limited to, natural bile acids found within the gut, the mouse gut microbiota, diet, and cage dominance. These variables as well as others will need to be explored in further research. In the meantime, the results of this study illustrate the general patterns of disease and inhibitory abilities of the bile salt analogs. CamSA can reduce or prevent CDI symptoms in mice for a few tested strains, while 07C is able to reduce, prevent, or reduce and delay CDI symptoms in mice with all but one strain tested. By modifying treatment while discovering new bile salt analogs, we may be able to find a potent prophylactic treatment option to be treated on CDI-inflicted organisms.
CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

4.1 Conclusions

CDI is an antibiotic-associated disease that is localized to the GI tract. After *C. difficile* spores become ingested through contact with various mediums such as table surfaces, food, animals, and unwashed hands, they undergo a transformative process while in the anaerobic intestines called germination. Bacterial cells that are capable of producing damage-inducing toxins arise from this germination process. CDI poses a serious threat to predisposed individuals who have weakened immune systems or who have recently taken antibiotics that deplete their natural protective bacterial barrier in the gut. With the emergence of new and hypervirulent strains of *C. difficile*, well individuals can also be vulnerable to CDI.

*C. difficile* spores germinate when in the presence of promoters called germinants. These germinants, specifically taurocholate, are bile acids that are naturally produced in the liver. Smaller amino acid co-germinants like glycine aid the germination process by increasing cooperative binding affinity of other co-germinants to the spore. Conversely, a few naturally occurring bile acids such as chenodeoxycholate can act as germination inhibitors. In individuals with gut microbiome imbalances, the germinant bile acids cannot be regulated efficiently by the indigenous intestinal microorganisms and inhibitor bile acids are not strong enough to protect from CDI in an overabundance of germinants.
Synthetic bile salt analogs have been proposed to act as more potent germination inhibitors than the natural inhibitor. One analog called CamSA was effective at preventing spore germination of strain 630 and CDI symptoms against a few other *C. difficile* strains in the CDI mouse model, while another analog called 07C was a powerful anti-germinant and CDI symptom reducer/preventer in multiple *C. difficile* strains. These bile salt analogs act as prophylactics against CDI and open the gateway to navigating the *C. difficile* germination pathway.

Germination assays were used to test bile salt analogs’ *in vitro* activity in preventing spore germination. CamSA was only able to prevent spore germination of strain 630 at less than 100 μM final concentration, while 07C inhibited germination of strains 630, R20291, 9001966, CDC 38, and DH1834 spores. Inhibitory activity could not be determined for strains 05-1223-046 and 7004578 as they were both unable to germinate in the presence of the natural activators taurocholate and glycine. No major germination profile similarities were found in different strains from the same ribotype group. The calculated IC$_{50}$ values from the germination assays provided a general outline of expectations for the *in vivo* study and were used for comparison purposes.

The bile salt analogs were tested as prophylactic agents against the various *C. difficile* strain spores *in vivo* in the CDI mouse model. Mice were challenged with an inoculum containing $10^8$ *C. difficile* spores via orogastric administration. Mice symptoms severity varied among each tested *C. difficile*
strain. Maximum CDI scores in untreated mice ranged from 4-8 on the day of maximal symptoms. Similarly, duration of disease symptoms also differed between strains. Interestingly, CamSA was not only able to protect mice from CDI when challenged with strain 630 spores, but it also prevented CDI symptoms in mice challenged with strains 05-1223-046 and 8085054, and reduced CDI symptoms in mice challenged with strains 9001966 and delayed symptoms in one mouse given strain 8085054. Overall, 07C was effective in preventing, reducing, or both reducing and delaying CDI in almost all tested strains.

Compound 07C protected mice challenged with 630, 05-1223-046, 7004578, and 8085054. Mice challenged with strain R20291 experienced a one day delay in symptoms onset, and the mean symptoms severity was significantly lower in the 07C-treated mice compared to the control. Delay of maximum symptom onset was also noted in mice that received strain 9001966 spores. DH1834-infected mice treated with 07C experienced a slight reduction in CDI symptoms and quick recovering soon thereafter. Of all the tested strains 07C seemed to be least effective against strain CDC 38 with only an insignificant reduction and delay in CDI symptoms.

The in vitro IC$_{50}$ values for each strain did not correlate with the degree of protection from CDI in vivo. However, the more potent compound, 07C, was generally a better prophylactic against multiple C. difficile strains than CamSA. The differences between in vitro and in vivo activities could be due to the influences that may only be present in living systems. A considerable amount of
heterogeneity in symptoms severity was found among mice of the same cage and treatment regimen. Although variables such as dosages, temperature, and light cycles were well controlled, other variables include ad libitum drinking of the antibiotic cocktail, mouse dominance, and epigenetic factors are more difficult to control.

4.2 Research Questions

The findings from both the in vitro and in vivo studies raise a few questions about variables that may confound results. To recap on peculiar discoveries from the in vitro study, two C. difficile strains, 05-1223-046 and 7004578, were unable to germinate in the presence of the germinants taurocholate and glycine. As previously mentioned in chapter 2, the appropriate amino acid co-germinant required for germination may not be the expected glycine. Alternatively, the co-germinant may be a molecule other than an amino acid. Once a suitable co-germinant is found, another question to answer is whether CamSA and 07C act as active inhibitors against those strains.

For the in vivo study, many possibilities may be responsible for the heterogeneity found within each group of mice. A recent article discussed the variability found between seemingly identical mice and associated it with minute and difficult-to-control factors.203 One proposed variable was sex. All mice used in the study were weaned female C57BL/6 mice. By the time mice were mature enough to be used in the study, they were of age to enter menarche (start of the estrous cycle). As most CDI mice studies are performed
using female mice, the variation between sexes was not yet considered. No current or previously published studies have explored the differences between male and female mice CDI thoroughly. One hallmark CDI mice study that happened to use both male and female mice showed similar symptoms severity between the two sexes.\textsuperscript{200}

Estrogen has been suggested to play a role in mediating gut inflammatory response and modifying intestinal permeability.\textsuperscript{204,205} Studies have found that post-menopausal women are at higher risk for intestinal diseases such as Crohn’s disease and Irritable Bowel Syndrome (IBS).\textsuperscript{206} Increased inflammatory bowel diseases (IBD) have also been associated with pregnancy and use of oral contraceptives.\textsuperscript{206,207} Furthermore, previous work from the Abel-Santos Laboratory showed that progesterone analogs affect \textit{in vitro} germination of \textit{C. difficile} spores.\textsuperscript{208} These results are intriguing since steroidal hormones share the same cholic backbone with bile salts.\textsuperscript{209} Therefore, regulation of estrogen via the estrous cycle may play a role in other intestinal diseases like CDI. Although estrogen’s role in Crohn’s and IBS have been well researched, estrous cycle effects on CDI have yet to be examined. Higher susceptibility to intestinal inflammation post-menopause may contribute to the increased CDI risk in elderly women. Conversely, changes in gut microbiota can also effect estrogen excretion and reuptake.\textsuperscript{205} Imbalances in intestinal microflora can cause an increase in \(\beta\)-glucuronidase, which can uncouple the normally excreted
estrogen-glucuronic acid complex, causing estrogen to be more readily reabsorbed by the gut, thus resulting in increased estrogen load.²¹⁰

We conducted a preliminary experiment to investigate differences between male and female CDI symptoms severity. The primary purpose of this experiment was to see if male mice experience more homogenous symptoms than female mice. Experimental setup was performed in the same manner as with other mouse CDI model experiments outlined in chapter 3. All cages were run in tandem. All mice were of the same age and were post-pubescent.

Although heterogeneity still existed, disease symptoms are statistically less severe in males than in female in the control groups (Figure 4.1, Table 14.1). Also, males seemed to have a more heterogenous spread than females (Figure 4.2). Of the five males in the control cage, two remained asymptomatic, while the other three had mild to moderate CDI. Interestingly, bile salt analogs failed to protect male mice from CDI. Although there have been several studies done on estrogen effects on several types of GI afflictions, testosterone effects have not been well characterized. It may still be advantageous to continue using female mice model; since male mice get slightly less sick from CDI, having female mice that are more susceptible to CDI may be important for observing minor changes in symptoms severity between regimens.
Figure 4.1. CDI symptoms severity in female mice challenged with strain 9001966 spores. Data points represent each animal’s symptoms severity based on the CDI scoring rubric. Overlap of data points occurs when animals share the same score. Error bars indicate standard deviation from the mean. Standard deviations represent a minimum of three independent values (n ≥ 3). Experimental groups were statistically significant (unpaired Student’s T-test) compared to the positive control group at p < 0.1 (*), p < 0.05 (**), and p < 0.01 (**). A) Symptoms severity of positive untreated control mice (○). B) Symptoms severity of mice treated with three doses of 50 mg/kg CamSA (□). C) Symptoms severity of mice treated with three doses of 50 mg/kg 07C (△).
Figure 4.2. CDI symptoms severity in male mice challenged with strain 9001966 spores. Data points represent each animal’s symptoms severity based on the CDI scoring rubric. Overlap of data points occurs when animals share the same score. Error bars indicate standard deviation from the mean. Standard deviations represent a minimum of three independent values (n ≥ 3). Experimental groups were statistically insignificant (unpaired Student’s T-test) compared to the positive control group up to p > 0.1. Males groups were statistically significant (unpaired Student’s T-test) compared to corresponding female groups at p < 0.1 (†), p < 0.05 (‡), and p < 0.01 (§). A) Symptoms severity of positive untreated control mice (○). B) Symptoms severity of mice treated with three doses of 50 mg/kg CamSA (□). C) Symptoms severity of mice treated with three doses of 50 mg/kg 07C (△).
Table 4.1. Comparison of mean CDI symptoms for all 9001966-challenged mice groups.

<table>
<thead>
<tr>
<th>9001966 Group</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
<th>96 h</th>
<th>120 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females Control</td>
<td>0</td>
<td>3.0</td>
<td>3.6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Males Control</td>
<td>0</td>
<td>2.4</td>
<td>1.8</td>
<td>1.0</td>
<td>0</td>
</tr>
<tr>
<td>Females with CamSA</td>
<td>0</td>
<td>0.4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Males with CamSA</td>
<td>0</td>
<td>2.2</td>
<td>1.6</td>
<td>0.6</td>
<td>0.2</td>
</tr>
<tr>
<td>Females with 07C</td>
<td>0</td>
<td>2.8</td>
<td>1.4</td>
<td>0.6</td>
<td>0</td>
</tr>
<tr>
<td>Males with 07C</td>
<td>0</td>
<td>2.8</td>
<td>1.4</td>
<td>0.2</td>
<td>0.2</td>
</tr>
</tbody>
</table>

**KEY:** light green = non-disease, green = non-diseased-to-mild CDI, yellow = mild CDI, yellow-orange = mild-to-moderate CDI, orange = moderate CDI

On a similar note, diet has also been shown to affect the diverse gut enterotypes. Thus, this may also have an effect of the prognosis of CDI. Long-term studies have shown that high levels of genus *Bacteroides* bacteria in the gut are linked to high protein and animal fat intake.\(^{211}\) Similarly, increase carbohydrate consumption is associated with genus *Prevotella* bacterial prevalence.\(^{211}\) Imbalance of these gut microbe could influence the progression of CDI.

Another uncertainty is whether symptom heterogeneity is caused by discrepancies in sporulation or in bile salt activation. As mentioned in section 1.2.2, differences in sporulation rates among various *C. difficile* strains may account for variation of symptoms onset and severity among mice. *In vivo* conditions may influence when spores germinate in the gut. One study using *C.*
difficile strain 630 revealed that spore shedding occurs over a 96-hour period from the time of infection.\textsuperscript{90} Therefore, strain 630 sporulation may occur up to that point. Moreover, recovered spores were undistinguishable from spore of the original inoculum, so it is possible that newer spores could be formed in the GI tract.\textsuperscript{90} These discoveries were only made for strain 630; other strains may behave differently in vivo.

At the bile salt level, cleavage of side chains in vivo by native gut microorganisms may also be probable. As a site of bile salt modification, the intestinal tract may also modify synthetic bile salt analog side chains. Therefore, finding a stable inhibitor is necessary. One study done by past members of the Abel-Santos Laboratory showed that CamSA was stable toward bile salt hydrolases.\textsuperscript{90}

\textbf{4.3 Future Directions}

There is a plethora of paths that can be explored to follow-up this thesis study. As this study is completed, other bile salt analogs are being tested as potential potent germination inhibitors. At this time, no other screened bile salt analog has shown \textit{in vitro} inhibitory activity as powerful as compound 07C.

To address the issue of other possible \textit{C. difficile} spore germinants, amino acid analogs will be tested with strains 05-1223-046 and 7004578 spores via germination assays. The most suitable germinant will be used to identify whether bile salt analogs CamSA and 07C demonstrate anti-germinants behavior.
Another matter to investigate is whether CamSA is an active inhibitor at higher concentrations. Although CamSA was not shown to be an active anti-germinant against five of the six testable C. difficile strains at low micromolar concentrations in vitro, CamSA was able to reduce CDI symptoms in mice given certain C. difficile strains. Also, IC₅₀ value was not found to have direct correlation to reduction of CDI symptoms possibly due to a number of in vivo factors to take into account. However, germination inhibition with CamSA in vitro may explain why it still exhibits prophylactic activity in mice that receive C. difficile strains other than strain 630.

Now that some results have been established in the prophylactic mice CDI model again various C. difficile isolates, we can adjust treatment regimens to cater to each individual strain. For some strains, upping the concentration of bile salt analog compound may necessary for protection from CDI. As no toxicity was observed at 300 mg/kg compound and only 50 mg/kg was given to C. difficile infected mice, increasing the concentration of bile salt analogs below 300 mg/kg should be non-toxic. Following that study, we can modify bile salt analog compound regimens to test on the extremely susceptible hamster model. As mentioned in chapter 3, CamSA protected hamsters from CDI when synergistically paired with vancomycin treatment. However, one animal did reach demise. By tailoring the treatment plans per strain, the potent inhibitor 07C may have effective prophylactic effects in the hamster model.
Further investigations into sex effects on CDI may be studied in more detail. If the estrous cycle may be a culprit in the heterogeneity of mice CDI symptoms, a vaginal epithelial swab can be used to determine the mice’s stage in the estrous cycle. One caveat to using this method is that it would be difficult to deliberately sync all mice in one cage to the same stage in the cycle. However, the stages could be used to backtrack and find possible correlations between them and CDI symptoms severity.

Issues concerning diet effects on gut microbiota can also be examined using the mice model. Changes in enterotype composition can be tracked as mice diet changes. Mice will be given different feeds containing an abundance of one or more types of macromolecules. Moreover, CDI susceptibility of mice on the special diets will be observed. Mice will then be treated with bile salt analog prophylactics while on the specific diets.

To address concern of bile salt analog absorption by intestinal epithelium or modification in the gut, an in vitro bind assay study will be used to test CamSA and 07C’s binding to the intestinal chyme. This will give us an understanding of how the absorptivity of these compounds by the intestinal epithelium. Moreover, the bile salt analogs’ effect on the gut microbiota can also be studied further.

Because onset of CDI symptoms occurred rapidly within 48 hours for the published study involving strain 630, bacterial loads following that timeframe could not be determined as the mice reached moribund. By using a non-toxigenic C. difficile strain, shedding spores from feces will be easier to recover
since mice will be asymptomatic. This also greatly minimizing possible suffering for the animal. Using a non-toxigenic strain also brings up the question of whether these non-toxigenic spores will still germinate in the gut like spores of other strains do if untreated.

CDI is a quick spreading quandary as new strains of C. difficile are still being discovered. Because of the variable responses of the strains to various environments and factors, finding a “one-size-fits-all” solution may not be possible for CDI. On top of that, there is also the conundrum that CDI is an antibiotic-associated disease treated by antibiotics. Therefore, the exploration into prophylactic treatment is indispensable. If these tested bile salt analogs can protect animals from CDI, they may be used to control the prognosis of CDI as molecular probes. This would lead CDI research one step closer to uncovering the binding action of these bile salt germinants and inhibitors to the spore. By focusing on the preventative capability of bile salt analogs like CamSA and 07C, we may be able to flush away this poopy problem in the near future.
Figure S1. Germination kinetic graph containing various concentrations of 07C against C. difficile strain 630 spores. These concentrations were used to construct the dose-dependent curve to determine the IC$_{50}$ of 07C against strain 630. Spores were resuspended in germination buffer and treated with neat DMSO (●) or treated with a fixed concentration of taurocholate (6 mM) and glycine (12 mM) added to final concentrations of 0 μM (●), 3.125 μM (●), 6.25 μM (●), 9.375 μM (●), 12.5 μM (●), 15.625 μM (●), 25 μM (●), 31.25 μM (●), 37.5 μM (●), 40.625 μM (●), and 50 μM (●) 07C. In actuality, data was collected every minute for 120 minutes. For clarity, data at five minute intervals are shown and error bars signifying standard deviation were eliminated. The data points indicate the means from three independent measures (n = 3).
Figure S2. Germination kinetic graph containing various concentrations of 07C against C. difficile strain R20291 spores. These concentrations were used to construct the dose-dependent curve to determine the IC$_{50}$ of 07C against strain R20291. Spores were resuspended in germination buffer and treated with neat DMSO (●) or treated with a fixed concentration of taurocholate (6 mM) and glycine (12 mM) added to final concentrations of 0 μM (●), 0.048828 μM (●), 0.146484 μM (●), 0.195313 μM (●), 0.390625 μM (●), 0.585938 μM (●), 0.78125 μM (●), 1.171875 μM (●), 1.5625 μM (●), 2.34375 μM (●), 6.25 μM (●), 9.375 μM (●), 15.625 μM (●), 25 μM (●), and 28.125 μM (●) 07C. In actuality, data was collected every minute for 120 minutes. For clarity, data at five minute intervals are shown and error bars signifying standard deviation were eliminated. The data points indicate the means from three independent measures (n = 3).
Figure S3. Germination kinetic graph containing various concentrations of 07C against C. difficile strain 9001966 spores. These concentrations were used to construct the dose-dependent curve to determine the IC\textsubscript{50} of 07C against strain 9001966. Spores were resuspended in germination buffer and treated with neat DMSO (●) or treated with a fixed concentration of taurocholate (6 mM) and glycine (12 mM) added to final concentrations of 0 μM (●), 1.5625 μM (●), 2.34375 μM (●), 3.125 μM (●), 6.25 μM (●), 9.375 μM (●), 15.625 μM (●), 18.75 μM (●), 25 μM (●), 31.25 μM (●), 37.5 μM (●), and 40.875 μM (●) 07C. In actuality, data was collected every minute for 120 minutes. For clarity, data at five minute intervals are shown and error bars signifying standard deviation were eliminated. The data points indicate the means from three independent measures (n = 3).
Figure S4. Germination kinetic graph containing various concentrations of 07C against *C. difficile* strain CDC 38 spores. These concentrations were used to construct the dose-dependent curve to determine the IC$_{50}$ of 07C against strain CDC 38. Spores were resuspended in germination buffer and treated with neat DMSO (●) or treated with a fixed concentration of taurocholate (6 mM) and glycine (12 mM) added to final concentrations of 0 μM (●), 0.1953125 μM (●), 0.390625 μM (●), 0.78125 μM (●), 1.5625 μM (●), 2.34375 μM (●), 3.90625 μM (●), 4.6875 μM (●), 6.25 μM (●), 9.375 μM (●), 12.5 μM (●), 15.625 μM (●), 25 μM (●), and 37.5 μM (●) 07C. In actuality, data was collected every minute for 120 minutes. For clarity, data at five minute intervals are shown and error bars signifying standard deviation were eliminated. The data points indicate the means from three independent measures (n = 3).
Figure S5. Germination kinetic graph containing various concentrations of 07C against C. difficile strain DH1834 spores. These concentrations were used to construct the dose-dependent curve to determine the IC$_{50}$ of 07C against strain DH1834. Spores were resuspended in germination buffer and treated with neat DMSO (●) or treated with a fixed concentration of taurocholate (6 mM) and glycine (12 mM) added to final concentrations of 0 μM (●), 0.78125 μM (●), 1.171875 μM (●), 1.5625 μM (●), 3.90625 μM (●), 4.6875 μM (●), 6.25 μM (●), 12.5 μM (●), 15.625 μM (●), and 18.75 μM (●) 07C. In actuality, data was collected every minute for 120 minutes. For clarity, data at five minute intervals are shown and error bars signifying standard deviation were eliminated. The data points indicate the means from three independent measures (n = 3).
Figure S6. Germination kinetic graph containing various concentrations of 07C against *C. difficile* strain 8085054 spores. These concentrations were used to construct the dose-dependent curve to determine the IC$_{50}$ of 07C against strain 8085054. Spores were resuspended in germination buffer and treated with neat DMSO (●) or treated with a fixed concentration of taurocholate (6 mM) and glycine (12 mM) added to final concentrations of 0 μM (●), 0.01220703125 μM (●), 0.0244140625 μM (●), 0.048828125 μM (●), 0.09765625 μM (●), 0.146484375 μM (●), 1.171875 μM (●), 1.5625 μM (●), 9.375 μM (●), 12.5 μM (●), 15.625 μM (●), 25 μM (●), 28.125 μM (●), 37.5 μM (●), 43.75 μM (●), and 50 μM (●) 07C. In actuality, data was collected every minute for 120 minutes. For clarity, data at five minute intervals are shown and error bars signifying standard deviation were eliminated. The data points indicate the means from three independent measures (n = 3).


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CURRICULUM VITAE

Graduate College
University of Nevada, Las Vegas

Jacqueline Renee Phan
jacquelinerphan@gmail.com

Degrees

Bachelor of Science, Biological Sciences, 2012
University of Nevada, Las Vegas

Bachelor of Science, Biochemistry, 2013
University of Nevada, Las Vegas

Special Honors and Awards

- 61st Annual Wind River Conference on Prokaryotic Biology Student and Post-Doctoral Travel Award (June 2017)
- UNLV Graduate & Professional Students Association (GPSA) Summer Travel Sponsorship Award (June 2017)
- 59th Annual Western Association of Graduate Schools (WAGS) Conference Three-Minute Thesis Competition 2nd Place Award (March 2017)
- 59th Annual Western Association of Graduate Schools (WAGS) Conference Three-Minute Thesis Competition People’s Choice Award (March 2017)
- 19th Annual UNLV Graduate & Professional Student Research Forum 2nd Place Award – Platform Session B (April 2017)
- UNLV Graduate Access Childcare Scholarship Award (November 2016)
- 3rd Annual UNLV Rebel Grad Slam Three-Minute Thesis Competition 1st Place Award (November 2016)
- Inaugural UNLV Graduate Research Showcase Scholarship Award (October 2016)
- 18th Annual UNLV Graduate & Professional Student Research Forum 2nd Place Award – Poster Session B (March 2016)

Publications

Oral and Poster Presentations


Outreach

- Student Panelist at UNLV Graduate College/GPSA Rebel Grad Slam Workshop, Las Vegas, Nevada (September 2017)
- Student Panelist at UNLV Graduate College Communications Certification Introductory Meeting, Las Vegas, Nevada (August 2017)
- Student Presenter and Panelist at UNLV Graduate College Preparing & Polishing your Presentation Skills Workshop, Las Vegas, Nevada (March 2017)
• GPSA Student Representative at the 79th Session of the Nevada Legislature, Carson City, Nevada (March 2017)
• Guest Speaker at UNLV Osher Lifelong Learning Institute (OLLI) Professor’s Choice Class, Las Vegas, NV (November 2016)

Thesis Title

A Potential Solution to a Poopy Problem: Bile Salt Analogs as Prophylactics Against Clostridium Difficile Infection

Thesis Examination Committee

Ernesto Abel-Santos, Ph.D., Advisory Committee Chair
Ronald K. Gary, Ph.D., Advisory Committee Member
Bryan L. Spangelo, Ph.D., Advisory Committee Member
Jefferson W. Kinney, Ph.D., Graduate College Representative