CLOSTPATH 13

Banff National Park, Canada
September 19-23, 2023

Artwork courtesy of Dr. Rita Tamayo

Welcome to ClostPath 13!

First held in Tuscon, AZ in 1995, the International Conferences on the Molecular Biology and Pathogenesis of Clostridia (ClostPath) bring together academics, clinicians, and industry scientists. Since its inception, the ClostPath conferences have been a popular forum to discuss the latest research and clinical findings regarding pathogenic Clostridia. Some of the original requirements to be categorized as a 'Clostridium' were that the organism was 1) Gram-positive, 2) an anaerobe, and 3) produced dormant endospores. Given that these criteria were based solely on phenotype and not genetics (as is now standard), much effort has gone into re-categorizing these organisms based off of sequencing. For that reason, much of the Clostridia have been renamed. However, their importance for human and animal health has not changed and several Clostridia have been a scourge of human health throughout history (e.g., an 1809 painting depicted a patient with a *Clostridium tetani* infection; tetanus). However, despite the availability of a vaccine to prevent tetanus, *C. tetani* infections continue to be problematic in IV drug users, diabetics, and approximately 30,000 newborns per year die from neonatal tetanus worldwide. Similarly, *Clostridium botulinum* is problematic in infants and has a 5% - 10% mortality rate if immediate treatment is not given. *Paeniclostridium* (formerly *Clostridium*) *sordellii* has recently gained attention as causing myonecrotic or enterotoxic disease in farm animals. In humans, *P. sordellii* can cause a near-universally fatal infection in postpartum women who become infected by this organism.

The ClostPath conferences are held every 2 – 3 years and their locations are decided upon by an Executive Steering Committee comprised of top researchers around the globe. This year, ClostPath 13 is held at the **Banff Centre for Arts and Creativity** in the gorgeous Banff, Alberta, Canada. The city of Banff is located adjacent to several Canadian Rocky Mountains Parks that are designated **UNESCO World Heritage Sites**. This year, we have 13 invited speakers and 33 oral presentations that were selected from submitted abstracts. Moreover, we have more than 100 posters that will be presented between two poster sessions. We are thankful to the **Executive Steering Committee** for helping us review all of the abstracts to choose the oral presentations. The task was not easy as there are stellar studies being conducted within our community and we're all exited to hear / see all of the stories! Of these oral presentations, 56% are from women and 61% are from junior investigators – thus continuing the strong tradition that ClostPath has of highlighting the research from junior scientists.

Speaking of Young Scientists, a Young Scientist meeting will again be held prior to the start of ClostPath 13. Began during ClostPath 11, the Young Scientist meeting allows networking with other trainees and hearing from panelists about different career paths. Thank you to **Annie Doyle** and **Morgan Osborne** for organizing this important meeting!

A special thank you **to Dr. Rita Tamayo** for, again, designing the amazing conference logo!

Finally, a huge thank you to the organizers of the ClostPath 12 conference: **Drs. Aimee Shen and Borden Lacy.** ClostPath 12 was originally planned to be held at the Banff Centre for Arts and Creativity but had to be transitioned to a virtual format at the last minute. Their herculean efforts in the ClostPath 12 conference have helped immensely with the planning for ClostPath 13. Looking forward to welcoming you to ClostPath 13!

Your organizers,

Joe Sorg, Casey Theriot, and Roman Melnyk

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CLOSTPATH 13 ORGANIZATION GROUP

CONFERENCE ORGANIZERS

Dr. Joe Sorg, Texas A&M University Department of Biology, USA
Dr. Casey Theriot, North Carolina State University, Department of Population Health and Pathobiology, USA
Dr. Roman Melnyk, University of Toronto, Research Institute, Hospital for Sick Children, Canada

INTERNATIONAL STEERING COMMITTEE

Dr. D. Borden Lacy, Chair (Professor, Vanderbilt University, USA).
Dr. Dena Lyras, Vice Chair (Professor, Monash University, Australia
Dr. Robert Fagan (Senior Lecturer, Sheffield University, UK).
Dr. Miia Lindström (Professor, University of Helsinki, Finland).
Dr. Bruce McClane (Professor, University of Pittsburgh, USA).
Dr. Kaori Ohtani (University of Kanazawa, Japan).
Dr. Daniel Paredes-Sabja (Associate Professor, Texas A&M University, USA).
Dr. Horst Posthaus (Professor, University of Bern, Germany).
Dr. Aimee Shen (Associate Professor, Tufts University, USA).
Dr. Wiep Klaas Smits (Assistant Professor, Leiden University, The Netherlands).
Dr. Rita Tamayo (Professor, University of North Carolina, USA).

YOUNG SCIENTIST COMMITTEE

Annie Doyle, University of Oklahoma Health Sciences Center, Department of Microbiology and Immunology

Morgan Osborne, Texas A&M University, Department of Biology

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TABLE 1: HISTORY OF THE CLOSTPATH CONFERENCES

Meeting	Year	Location	Attendees	Highlights
ClostPath 1	1995	Tucson, AZ	125	Publication of the major resource "The Clostridia: Molecular Biology and Pathogenesis
ClostPath 2	1997	Seillac, France	120	Structure of the botulinum neurotoxins; MOA of *C. difficile* toxins (TcdA and TcdB)
ClostPath 3	2000	Chiba, Japan	110	Genome sequencing of major Clostridial pathogens (*C. perfringens* & *C. difficile*)
ClostPath 4	2003	Woods Hole, MA	127	Completed genome sequences of *C. tetani* and *C. perfringens*
ClostPath 5	2006	Nottingham, England	175	First demonstration of a genetic system for *C. difficile* (group II introns)
ClostPath 6	2009	Rome, Italy	206	Use of genetic systems to probe the importance of *C. difficile* toxins in an animal model of disease
ClostPath 7	2011	Ames, IA	152	Keynote on cholesterol-dependent cytolysins
ClostPath 8	2013	North Queensland, Australia	161	First report of LSR as a receptor for *C. perfringens* TpeL toxin; First report of a germinant receptor for *C. difficile* spores.
ClostPath 9	2015	Freiburg, Germany	195	Crystal structure of *C. difficile* TcdA
ClostPath 10	2017	Ann Arbor, MI	223	*C. difficile* phase varies several of its virulence determinants; keynote on metabolism & gene expression in *C. difficile*
ClostPath 11	2019	Leiden, The Netherlands	230	Identification of *C. sordellii* host receptor for the TcsL toxin; First Young Scientist Meeting
ClostPath 12	2021	Virtual	442	Impact of bile acids on *C. difficile* toxin function; How nutrient competition shapes *C. difficile* infection

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We'd like to thank the generous Gold-level sponsorship from Cerillo Bio. Cerillo's portable plate reader is helping scientists anticipate and creatively solve human challenges from the palm of their hand, giving them the freedom to take their work out into the world. Please visit Cerillo at ClostPath 2023 to hear more about what they can offer.

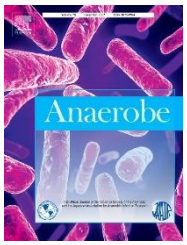
We'd like to thank Ferring Pharmaceuticals for their generous Gold-level sponsorship of ClostPath 13. Ferring is a research-driven, specialty biopharmaceutical group committed to helping people build healthy families and live better lives. Ferring is a leader in reproductive medicine and maternal health, and in specialty areas within gastroenterology and urology. Please see their website for more details.

We'd like to thank the generous Bronze-level sponsorship from Vedanta Biosciences. Vedanta rationally designs medicines based on consortia of human commensal bacteria to treat disease, using insights from microbial ecology, mucosal immunology, and human interventional studies. For more information about how Vedanta Biosciences is rationally designing microbiome-based therapies, please visit their website.

We'd like to the generous Bronze-level sponsorship from Microbiology International. Microbiology International was formed in 1997 to provide the best microbiology automation from around the world to the modern laboratory. Their equipment quickly automates tedious, manual lab processes and when combined with the ability to provide customized culture media products, and pathogen ID kits. For more information on Microbiology International, please visit their website. At ClostPath 13, Microbiology International will be exhibiting a scientific instrument by their business partner, Don Whitley Scientific. Don Whitley Scientific Limited develops, manufactures and sells equipment and associated products for microbiology and cell culture applications worldwide. All products designed and developed by DWS are manufactured in our own premises in England.

We'd like to thank Anaerobe Systems for their Bronze-level sponsorship! Anaerobe Systems was founded in 1978 by Mike Cox. Anaerobe Systems produces the world's only true pre-reduced anaerobically sterilized (PRAS) culture media, developed and patented the first gloveless anaerobic chamber over 40 years ago and ever since has been closely involved in anaerobe chamber design, development, and functionality, and is committed to furthering education and training in anaerobic bacteriology. Their company conducts a *3-day hands on lab based anaerobic bacteriology course* five times per year and include that course free of charge for anyone purchasing our anaerobic chambers. Please see their website for more details.

TECHLAB designs, develops, and manufactures enteric diagnostics that are distributed worldwide. TechLab offers diagnostic tests for *C. difficile*, parasitology, intestinal inflammation, Shiga toxins, & *Campylobacter.* Check out their website for more details!



Anaerobe is essential reading for those who wish to remain at the forefront of discoveries relating to life processes of strictly anaerobes. The journal is multi-disciplinary, and provides a unique forum for those investigating strictly anaerobic organisms that cause infections in humans and animals, as well as anaerobes that play roles in microbiomes or environmental processes. **A special issue of *Anaerobe* will be published based upon abstracts / presentations at ClostPath 13. Stay tuned!**

We would like to thank the generous sponsorship from North Carolina State University, College of Veterinary Medicine. Ranked among the best in the nation, the NC State College of Veterinary Medicine is a driving force in veterinary research and training. Please see the link above for more information.

Code of Conduct for the 13th International Conference on the Molecular Biology and Pathogenesis of Clostridia (ClostPath 2023).

The ClostPath conference series has always been a welcoming environment for diverse ideas and has aimed to create a respectful and inclusive environment that promotes collaboration, scientific integrity, and meaningful discussions. As stated in NIH policy, Civil Rights Protections in NIH-Supported Research, Programs, Conferences and Other Activities, consistent with existing federal civil rights laws, it is expected that organizers of NIH-supported conferences and scientific meetings take steps to maintain a safe and respectful environment for all attendees by providing an environment free from discrimination and harassment, sexual or otherwise. A detailed Code of Conduct can be found on the ClostPath 13 website.

Safety: Local police information can be found: https://banff.ca/178/Police---RCMP. In an emergency, please dial 911.

Federal Funding Acknowledgment:

Funding for this conference was made possible, in part, by 1R13AI178883 from the United States National Institutes of Health / National Institute of Allergy and Infectious Diseases (NIAID). The views expressed in written conference materials or publications and by speakers and moderators do not necessarily reflect the official policies of the Department of Health and Human Services; nor does mention of trade names, commercial practices, or organizations imply endorsement by the U.S. Government.

Funding for this conference was made possible, in part, by PCS-189451 from the Canadian Institutes for Health Research Institute for Infection and Immunity: Planning and Dissemination Grant

September 19

Time			
13:30 – 16:30	Young Scientist Meeting		
17:00 – 18:30	Welcome Dinner		
19:00 – 20:00	Keynote Address	Dr. Aimee Shen	Of Spores and Cells: Insights into the Evolution of Cell Division Mechanisms of *Clostridioides difficile*

September 20

Session I: Microbe-Microbe Interactions (Anna Seekatz / Jacqueline Phan)		
Time	**Speaker**	**Title**
9:00 – 9:30	Dr. Joe Zackular	Mechanisms of interaction between *Clostridioides difficile* and enterococci
9:30 – 9:45	Samantha Kisthardt	Stickland amino acid availability alters the expression of the bile acid inducible (*bai*) operon in commensal Clostridia
9:45 – 10:00	Dr. Nian Liu	*Clostridium septicum* and *C. difficile* compete for an intestinal lipid-rich environment lacking in naturally evolved antimicrobial activity
10:00 – 10:15	Rebbekah Menday	How Cryo-EM structures of a whole contractile phage can begin to demonstrate mechanisms of phage infection
10:15 – 10:30	Break	

Session II: One Health (Horst Posthaus / Annie Doyle)		
Time	**Speaker**	**Title**
10:30 – 11:00	Dr. Luis Arroyo Castro	Clostridial diseases of large animal species: old and new
11:00 – 11:15	Dr. Francisco Uzal	Experimental acute *Clostridium perfringens* type D enterotoxemia in sheep is not characterized by specific renal lesions
11:15 – 11:30	Dr. Stuart Johnson	Enteritis Necroticans due to *Clostridium perfringens* type C; Epidemiological and pathological findings over the past 20 years
11:30 – 11:45	Natasza Hain-Saunders	*Clostridioides difficile* in feral horse populations in Australia
11:45 – 13:30	Lunch	

Session III: -Omics (Wiep Klaas Smits / Samantha Kisthardt)		
Time	**Speaker**	**Title**
13:30 – 14:00	Dr. Olga Soutourina	RNomics during *C. difficile* infection cycle: from identification to function
14:00 – 14:15	Manuela Fuchs	Hfq RIL-seq in *C. difficile* reveals sRNA-mediated regulation of sporulation
14:15 – 14:30	Jessical Buddle	Rapid evolution under vancomycin selection reveals multiple pathways to resistance in *Clostridioides difficile*
14:30 – 14:45	Dr. Nick Markham	A single-cell resolution, multi-omic spatial atlas of colonic tumorigenesis driven by *C. difficile* from human colorectal cancer-associated biofilms
14:45 – 15:00	Break	

Session IV: Treatments / Healthcare (Michael Abt / Ajisha Alwin)		
Time	**Speaker**	**Title**
15:00 – 15:30	Dr. Larry Kociolek	Recent developments in the treatment and prevention of *C. difficile* infection
15:30 – 15:45	Anna DeVeaux	Functional microbiome variation influences *Clostridioides difficile* infection susceptibility
15:45 – 16:00	Dr. Wiep Klaas Smits	*Clostridioides difficile* PCR ribotype 151 is polyphyletic and includes pathogenic isolates from cryptic clade C-II with mono-toxin pathogenicity loci that can escape routine diagnostics
16:00 – 16:15	Dr. Mayland Chang	A dual-action antibiotic that kills *Clostridioides difficile* vegetative cells and inhibits spore germination
16:15 – 16:30	Break	

Session V: Physiology (Rita Tamayo / Katherine Wozniak)		
Time	**Speaker**	**Title**
16:30 – 17:00	Dr. Erin Purcell	Alarmone Signaling in *C. difficile*: Newly discovered variations on a conserved pathway
17:00 – 17:15	Dr. Lynn Bry	HRMAS 13C NMR and genome-scale metabolic modeling identify threonine as a preferred dual redox substrate for *Clostridioides difficile*
17:15 – 17:30	Dr. Hualiang Pi	Iron Storage Organelles Facilitate *Clostridioides difficile* Survival in the Gut
17:30 – 17:45	Dr. Jihong Li	Contact of *Clostridium perfringens* type A strain ATCC3624 with C2C12 muscle cells increases toxin production to enhance ATCC3624 growth in a process involving the EutV/W two component system
18:15 – 19:30	Poster Session I & Reception	
19:30 –	Dinner on your own	

September 21

Session VI: Microbiome (Vincent Young / Anna DeVeaux)		
Time	**Speaker**	**Title**
8:30 – 9:00	Dr. Casey Theriot	Reengineering of the gut bile acid landscape to restrict *C. difficile*
9:00 – 9:30	Dr. Thomas Louie	A solid movement with RBX2660. Safely restoring gut microbiota from donor to patient
9:30 – 9:45	Dr. James Collins	Control of *C. difficile* Toxins and Repair of the Gut Endothelium by a Microbial Metabolite
9:45 – 10:00	Break	

Session VII: Dr. Tohru Shimizu Memorial Lecture (Joe Sorg)		
Time	**Speaker**	**Title**
10:00 – 10:45	Dr. Bruce McClane	With a Little Help from My Friends: *Clostridium perfringens* Quorum Sensing

Session VIII: Toxins (Borden Lacy / John Manion)		
Time	**Speaker**	**Title**
10:45 – 11:15	Dr. Dena Lyras	Non-antibiotic strategies to mitigate *C. difficile* infection: insights from discovery research
11:15 – 11:30	Dr. Andreas Rummel	Presynaptic targeting of botulinum neurotoxin type A requires a tripartite PSG-Syt1-SV2 plasma membrane nanocluster for synaptic vesicle
11:30 – 11:45	Dr. Stephen Melville	Holin-dependent secretion of the large clostridial toxin TpeL by *Clostridium perfringens*
11:45 – 13:15	Lunch	
12:45 -	Planned Activities / Free time / Dinner on your own **Planned Excursion** Pickup location: Banff Centre - PDC Building (Home for Arts and Creativity) Pickup time: 12:45pm Tour start time: 1:00pm Drop off time: 5:30pm	

September 22

Session IX: Spores (Aimee Shen / Morgan Osborne)		
Time	**Speaker**	**Title**
8:30 – 9:00	Dr. Marjorie Pizarro-Guajardo	Understanding surface variability in *Clostridioides difficile* spores and implications in disease.
9:00 – 9:15	Dr. Hannah Fisher	Spore Ultrastructure and Germination in *Clostridium sporogenes*
9:15 – 9:30	Dr. Paula Salgado	*C. difficile* engulfasome at the molecular level
9:30 – 9:45	Morgan McNellis	Pseudoprotease-mediated regulation of germinant sensing in *Clostridioides difficile*
9:45 – 10:00	Break	

Session X: Gene Regulation (Erin Purcell / Merilyn Beebe)		
Time	**Speaker**	**Title**
10:00 – 10:30	Dr. Craig Ellermeier	Identification of a Two-Component Signal Transduction System That Responds to Lipid II-Interacting Antibiotics
10:30 – 10:45	Dr. Iman Mehdizadeh Gohari	Identification of orphan histidine kinases that impact sporulation and enterotoxin production by *Clostridium perfringens* type F strain SM101 in a pathophysiologically-relevant ex vivo mouse intestinal contents model
10:45 – 11:00	Léo Caulat	The armada of oxidative stress detoxication enzymes of *C. difficile*
11:00 – 11:15	Dr. Adrianne Edwards	The RgaSR two-component system promotes *Clostridioides difficile* sporulation through a small regulatory RNA and the AgrD1 autoinducing peptide
11:30 – 13:00	Lunch	
13:00 – 14:30	Poster Session II	

Session XI: Virulence Factors (Young Scientist Session) (Bruce McClane / Cyril Anjou)		
Time	**Speaker**	**Title**
14:30 – 14:45	Matthew Munneke	*Clostridioides difficile* nucleobase scavenging in the competitive gut environment
14:45 – 15:00	Dr. Jacqueline Phan	Of Mice and Women: The Effect of the Estrous Cycle on *Clostridioides difficile* Infection Outcomes
15:00 – 15:15	Dr. Sean Miletic	Structural characterization of bile acid inhibition of *Clostridioides difficile* Toxin B

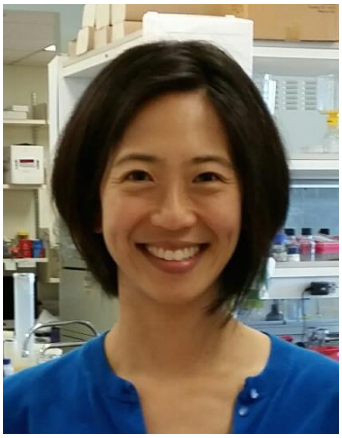
15:15 – 15:30	D. Annie Doyle	*C. difficile* TcdB-receptor tropism and binding interactions are driven by extracellular calcium
15:30 – 15:45	Clara Bate	Understanding the role of a key sporulation specific protein in beta-lactam resistance in *Clostridioides difficile*
15:45 – 16:00	Break	

Session XII: Host Response to Infection (Jimmy Ballard / Chris Peritore-Galve)		
Time	**Speaker**	**Title**
16:00 – 16:30	Dr. Michael Abt	Mechanisms of immune modulation to support microbiome-based therapeutics
16:30 – 16:45	Kaylee Norman	*Clostridioides difficile* Toxin B subverts B cell migration, germinal center formation and antibody class switching following vaccination
16:45 – 17:00	Alex Huber	Transcriptional landscape of *Clostridioides difficile* infection-induced neutrophilia
17:00 – 17:15	Ashleigh Rogers	*Clostridioides difficile* infection disrupts host colonic repair
19:00 –	Congress Dinner	

Of Spores and Cells: Insights into the Evolution of Cell Division Mechanisms of *Clostridioides difficile*

Aimee Shen[1]

[1]Tufts University School of Medicine



Aimee Shen performed her Ph.D. work in microbiology with Dr. Darren Higgins at Harvard Medical School studying the regulation of flagellar gene expression in *Listeria monocytogenes.* She performed her postdoctoral work in chemical biology with Dr. Matthew Bogyo at the Stanford School of Medicine, where she studied the mechanism by which a protease domain found in bacterial toxins is allosterically activated by a eukaryotic-specific small molecule using structural biology and activity-based probes. While she studied the proteolytic activation of glucosylating toxins in *Clostridioides difficile* during her postdoctoral work, when she started her lab at the University of Vermont in 2011, she shifted the focus of her research to investigating how *C. difficile* forms infectious spores and then germinates these spores to initiate infection. In 2016, her lab relocated to the Tufts University School of Medicine. Using genetic, biochemical, cytological, and structural methods, her lab has identified and characterized novel regulators of both these developmental processes and contributed to a growing body of work that highlights the diversity of mechanisms by which Firmicutes build and germinate spores. Through this work on sporulation, her group has started to study mechanisms by which *C. difficile* grows and divides vegetatively including during stressful conditions using methods like chemical proteomics and time-lapse microscopy. Her lab has also started developing fluorescent reporters for studying *C. difficile* physiology during these conditions.

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Mechanisms of interaction between *Clostridioides difficile* and enterococci

Joe Zackular[1]

[1]Perelman School of Medicine at the University of Pennsylvania

Joe Zackular is an Assistant Professor in the Department of Pathology and Laboratory Medicine at the University of Pennsylvania and the Children's Hospital of Philadelphia. He received his PhD from the University of Michigan where he studied the role of the gut microbiota in colorectal cancer in the laboratory of Dr. Patrick Schloss. He trained with Dr. Eric Skaar at Vanderbilt University Medical Center for his postdoctoral fellowship where he studied the role of dietary metals and nutritional immunity in *Clostridioides difficile* infection. The Zackular laboratory is focused on understanding how interactions between the host, gut microbiota, and pathogenic microbes impact human health and disease. The labs recent efforts center on understanding how the important nosocomial pathogen, *C. difficile*, interacts with resident gut microbiota during infection and how interspecies cross-talk impacts infection. Research in the Zackular lab draws from a number of diverse fields including microbial ecology, bacterial pathogenesis, biochemistry, host-pathogen interactions, and microbiota research.

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Stickland amino acid availability alters the expression of the bile acid inducible (*bai*) operon in commensal Clostridia

Samantha Kisthardt[1], Arthur McMillan[1], Casey Theriot[1]

[1]North Carolina State University, College of Veterinary Medicine, Department of Population Health and Pathobiology

Clostridium scindens is a commensal that metabolizes primary bile acid cholate (CA) into secondary bile acid deoxycholate (DCA), which are inhibitory to *Clostridioides difficile*. Recent work showed that the *C. scindens bai* operon decreased expression in the presence of the proline precursor hydroxyproline. Proline and glycine are important for Stickland fermentation in both commensal *Clostridia* and the pathogen *C. difficile*. The consumption of these amino acids by *C. scindens* provides competition, further contributing to the inhibition of *C. difficile* by an intact gut microbiota. We hypothesize that the availability of amino acids used for Stickland fermentation alters the expression of the *bai* operon in commensal *Clostridia,* ultimately controlling secondary bile acid metabolism. To test this, we grew *C. scindens* in excess proline or glycine, in the presence and absence of CA or DCA. At mid-log growth, supernatant was collected for RNAseq and metabolomic analysis. Supplementation of CA significantly increased expression of the *bai* operon in *C. scindens*. Supplementing proline and CA maintained the same magnitude of expression, while the addition of glycine significantly decreased expression. Proline and CA altered the global transcriptome with 270 genes being differentially expressed, compared to only 50 genes with glycine. Genes important for carbohydrate metabolism, proline usage (*prd*), and cellular energetics in the form of molybdenum transfer and biosynthesis increased, while genes involved in the synthesis of proline and arginine metabolism (*pro* and *arg*) decreased. These findings suggest an avenue to modulate secondary bile acid production in commensal *Clostridia* with amino acid supplementation via diet.

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Clostridium septicum and *C. difficile* compete for an intestinal lipid-rich environment lacking in naturally evolved antimicrobial activity

Nian Liu[1], Serge Fobofou[1], Nazli Yalcinkaya[1], Sik Yu So[1], Shyam Badu[1], Margaret Conner[2, 3], Qinglong Wu[1], Tor Savidge[1]

[1]Department of Pathology & Immunology, Baylor College of Medicine, Houston, Texas, USA, [2]Department of Molecular Virology and Microbiology, [3]Department of Education, Innovation and Technology, Baylor College of Medicine, Houston, Texas, USA

Clostridium septicum is a gram-positive, toxin-producing bacterium known to cause gas gangrene. Infection is thought to involve hematogenous dissemination from the intestine, although colonization rates in humans remain poorly understood. To investigate susceptibility risks linked to *C. septicum* colonization, we conducted an extensive metagenomic survey of over 35,000 healthy and patient fecal specimens and observed that *C. septicum* in adults was exceptionally rare. However, intriguingly, this organism was commonly detected in infants. Furthermore, in-depth metagenomic characterization of the infant gut microbiome revealed a shared community representation between *C. septicum* and *C. difficile* colonization. Notably, the absence of shared taxa in this context conferred protection in experimental models of *C. difficile* infection. To substantiate the existence of a competitive gut niche shared by these diverse pathogens, we established an oral model of *C. septicum* infection. We demonstrated that asymptomatic carriage of *C. difficile* completely abrogated clinical disease caused by toxigenic *C. septicum*. Employing an integrated multi-omics approach, we identified a specialized intestinal niche, enriched in ceramides, sphingolipids, and sterols, that is commonly inhabited by these pathogens. Particularly, ceramides represent a key intermediate in sphingolipid biosynthesis known to facilitate host-pathogen interactions. Through comprehensive metagenomic comparisons and bioactivity-guided analysis of key microbiota taxa associated with pathogen decolonization, we successfully identified and elucidated the structure of hadromycin, a novel ether-class of sphingolipid possessing broad antimicrobial activity against *C. septicum* and *C. difficile*. Thus, sphingolipids have evidently evolved in the natural design of clostridial pathogen evasion during human development.

Acknowledgements: Gut Check Foundation, P01-AI152999, U01-AI24290

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How Cryo-EM structures of a whole contractile phage can begin to demonstrate mechanisms of phage infection

Rebbekah Menday[1], Jason Wilson[2], Louis-Charles Fortier [3], Robert Fagan[1], Per Bullough[1]

[1]University of Sheffield, [2]University of York, [3]Université de Sherbrooke

Bacteriophage-based therapies are a promising strategy for treating *Clostridioides difficile*, but exploitation will require a deeper understanding of the mechanism of infection. Structures of intact phages are required to understand the mechanism of phage infection and to find sites for engineering in these very diverse particles. Phage structures are highly complex, with multiple components, and are typically very large (>15-20 MDa) making structure determination enormously challenging. This may be one of the factors contributing to the lack of understanding surrounding their mechanism of infection. The *C. difficile* cell is coated with a crystalline protein array known as the S-layer, the structure of which was recently solved. As very few phages that infect S-layer-containing bacteria have been studied, it is not known how phages interact with, bind to and penetrate the S-layer before spanning the underlying cell wall and membrane. Consequently, our work focuses on understanding the 3D structural molecular detail of phage binding and penetration of this multi-layered cell envelope. To achieve this, we have determined the structures of selected *C. difficile* myoviruses using Cryo-EM and single particle analysis. This has led to the discovery of enzymatic domains, diverse needle architectures and contraction types that hint at a previously uncharacterised mechanism of phage infection.

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Clostridial diseases of large animal species: old and new

Luis Arroyo Castro[1]

[1]The University of Guelph

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Luis trained as a veterinarian at the Universidad Nacional-Heredia, Costa Rica. After graduation he practiced in rural Costa Rica for a few years and in 2000 he moved to Guelph to enroll in the large animal medicine Doctor of Veterinary Science (DVSc) program in the Department of Clinical Studies, at the Ontario Veterinary College, University of Guelph. He later completed a PhD in the Department of Pathobiology at the same institution and joined the Department of Clinical studies as an assistant professor in 2009. He is board certified in large animal medicine with the American College of Veterinary Internal Medicine. Luis research interest focus is in large animal gastrointestinal disorders, in particular enterocolitis in horses.

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Experimental acute Clostridium perfringens type D enterotoxemia in sheep is not characterized by specific renal lesions

Francisco Uzal[1], Federcio Giannitti[2], Vicki Adams[3], Joaquin Armenfano[4], Julian Rood[3], Juliann Beingesser[1]

[1]UCDavis, [2]Instituto Nacional de Investigación Agropecuaria, Uruguay, [3]Monash University, Australia, [4]Universidad Nacional del Centro de la Provincia de Buenos Aires, Argentina

Type D enterotoxemia, caused by *Clostridium perfringens* epsilon toxin (ETX), is one of the most economically important clostridial diseases of sheep. Acute type D enterotoxemia is characterized by well-documented lesions in the nervous, cardiocirculatory, and pulmonary systems. However, discrepancies and confusion exist as to whether renal lesions are part of the spectrum of lesions of this condition, which is controversial considering that for many decades it has been colloquially referred to as "pulpy kidney disease." Here, we assess renal changes in an experimental model of acute type D enterotoxemia in sheep and evaluate the possible role of ETX in their genesis. Four groups of 6 sheep each were intraduodenally inoculated with either a wild-type virulent *C. perfringens* type D strain, an *etx knockout* mutant unable to produce ETX, the *etx* mutant strain complemented with the wild-type *etx* gene that regains the ETX toxin production ability, or sterile culture medium (control group). All sheep were autopsied <24 hours after inoculation; none of them developed gross lesions in the kidneys. Ten predefined histologic renal changes were scored in each sheep. The proportion of sheep with microscopic changes and their severity scores did not differ significantly between groups. Mild intratubular medullary hemorrhage was observed in only 2 of the 12 sheep inoculated with the wild-type or *etx*-complemented bacterial strains, but not in the 12 sheep of the other 2 groups. The authors conclude that no specific gross or histologic renal lesions are observed in sheep with experimental acute type D enterotoxemia.

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Enteritis Necroticans due to Clostridium perfringens type C; Epidemiological and pathological findings over the past 20 years

Stuart Johnson[1, 2], Andrew Skinner[1, 2]

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Enteritis necroticans (EN) was originally described in the Highlands of Papua New Guinea (PNG). The etiologic agent of EN is beta toxin-producing *C. perfingens* type C (CPC). We reviewed the literature since January 2000 for reports EN in humans, and animal diseases linked to CPC.

Although not as prevalent in PNG as before the 1980's when beta toxoid vaccination was introduced, cases still occur in the Highlands. Outside of PNG, EN was confirmed in 5 reports from Japan, the U.K., the U.S., and Sri Lanka by recovery of CPC or beta toxin gene amplification from involved tissues. CPC was also suspected in 7 additional reports of EN from the U.S., Japan, Belgium, Switzerland, India, and Bangladesh based on pathological and microbiological findings. The case series from the South Asia included young children similar to the cases from PNG whereas reports from elsewhere include adults, association with diabetes mellitus, and consumption of a suspected food source prior to symptom onset. CPC has also been associated with necrotizing enteritis in piglets, foals, cattle, and goats as well as lamb dysentery (struck). In addition to clinical cases of EN, CPC has been isolated from numerous clinical and environmental samples worldwide including stools of infants in Jordan, freshwater fish in China, farm animals in Saudi Arabia, and horse fecal and soil samples from South Korea.

EN remains an enigmatic disease due to beta toxin-producing CPC with a world-wide distribution and has the potential for outbreaks as well as isolated cases.

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Clostridioides difficile in feral horse populations in Australia

Natasza Hain-Saunders[1], Daniel Knight[2], Andrea Harvey[3], Mieghan Bruce[1], Thomas Riley[4]

[1]Murdoch University, Western Australia, [2]Pathwest Laboratory Medicine, [3]University of Technology Sydney, [4]University of Western Australia

Clostridioides difficile is a known cause of diarrhoea and colitis in human and non-human animals. While *C. difficile* is regularly isolated from domesticated horses, little is known about prevalence in wild or feral populations. In Australia, the horse population encompasses a mix of both domesticated and feral animals. This study investigated the presence and characteristics of *C. difficile* in Australian feral horses and evaluated their potential as a source/reservoir of *C. difficile* in the wider community.

Faecal samples (n=380) were collected from free-roaming feral horses from five states across Australia and cultured for *C. difficile* by enrichment methods. Isolates were characterised by PCR ribotyping and toxin profiling, and antimicrobial susceptibility testing was performed by an agar incorporation method. Closed reference genomes were generated for two novel toxigenic strains, of sequence-type 963 and 964.

C. difficile was isolated from 45 of the 380 samples (11.8%), a significantly lower proportion than recent studies on Australian domesticated horses (P<0.001), however, like other wild animal species worldwide. Eighteen toxigenic *C. difficile* strains were isolated, eight containing binary toxin genes. Forty ribotypes were identified, 28 of which (70%) were novel; other ribotypes had been seen previously in humans, livestock and soils. Strains were largely susceptibility to the 10 antimicrobial agents tested.

This investigation provides preliminary information on the epidemiology of *C. difficile* infection in feral horses in Australia and allows a comparison with their domestic counterparts. The findings support the hypothesis that all horse faeces represent a potential reservoir of *C. difficile* in the community.
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RNomics during *C. difficile* infection cycle: from identification to function

Olga Soutourina[1]

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Olga Soutourina is a Professor in Microbiology at the Paris-Saclay University, Co-supervisor of Fundamental Microbiology M2 in Life Sciences and Health Master Program, HDR diploma Advisor in Genetics, Genomics and Microbiology in Paris-Saclay University, a group leader and deputy director of the Microbiology department in the Institute for Integrative Biology of the Cell (I2BC), France. Throughout her research activities she showed a constant interest for the regulation of gene expression in bacteria and for the understanding of molecular mechanisms of these regulatory processes involving pleiotropic proteins and regulatory RNAs. She prepared a Ph.D. in Molecular Genetics on the mechanisms controlling bacterial motility at Pasteur Institute in Paris, France. After a postdoctoral experience as an Assistant Lecturer in Biology department of the *Ecole Polytechnique* and in CNRS, she has obtained an Assistant Professor position in Paris Diderot University and worked at Pasteur Institute in Paris on the sulfur metabolism control in Firmicutes and initiated new projects on the RNA-based control in Clostridia as a group leader. She has received a prestigious award from French University Institute IUF and leads now a group at I2BC that works on the regulatory RNAs in Clostridia. The recent contributions of the team to the regulatory RNA field come from the genome-wide identification of non-coding RNAs in an important human pathogen *Clostridioides* (*Clostridium) difficile*, the analysis of RNAs interacting with RNA chaperone protein Hfq, the first evidence for CRISPR RNA expression and defensive function of *C. difficile* CRISPR-Cas system and the identification of functional antisense RNAs within type I toxin-antitoxin systems in this pathogen. The team also develops new genome editing tools based on endogenous CRISPR-Cas and toxin-antitoxin systems.

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Hfq RIL-seq in *C. difficile* reveals sRNA-mediated regulation of sporulation

Manuela Fuchs[1, 2], Vanessa Lamm-Schmidt[1, 2], Tina Lenče[1], Johannes Sulzer[1], Janet Wackenreuter[2], Arne Bublitz[3], Milan Gerovac[1], Till Strowig[3, 4], Franziska Faber[1, 2]

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The anaerobic, spore forming human pathogen *Clostridioides difficile* is the leading cause of antibiotic-associated diarrhea. Intestinal persistence and transmission of *C. difficile* occurs *via* antibiotic resistant spores, ingested by the host. Despite the importance of sporulation for *C. difficile* pathogenesis, molecular mechanisms regulating sporulation initiation remain ill defined.

To identify novel post-transcriptional regulatory mechanisms of spore formation, we performed RIL-seq (RNA interaction by ligation and sequencing) with Hfq, a global RNA binding protein. We discovered two Hfq-associated small regulatory RNAs (sRNAs), SpoX and SpoY, that interact with Spo0A, the master regulator of sporulation, by binding the 5'UTR of the *spo0A* mRNA. Interestingly, these interactions modulate translation of *spo0A* with opposite effects. While SpoX increases Spo0A protein levels, binding of SpoY to the *spo0A* 5'UTR leads to a decrease of Spo0A. Both interactions ultimately result in overall changes in sporulation frequencies on the population level *in vivo*. Furthermore, infection of antibiotic-treated mice with SpoX and SpoY deletion mutants revealed a global effect on gut colonization and intestinal sporulation. Taken together, our work uncovers a complex post-transcriptional layer in the regulation of sporulation initiation with implications for host infection and transmission of this important human pathogen.

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Rapid evolution under vancomycin selection reveals multiple pathways to resistance in *Clostridioides difficile*

Jessica E Buddle[1], Claire E Turner[1], Roy R Chaudhuri[1], Robert P Fagan[1], Michael A Brockhurst[2]

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Clostridioides difficile is an urgent threat to global health, owing, at least in part, to its widespread resistance to a multitude of antibiotics. Consequently, treatment relies largely upon just two antibiotics, vancomycin and fidaxomicin. Despite being the front-line antibiotic in the UK, little is known about the routes to vancomycin resistance in *C. difficile.* This project therefore aimed to genetically and phenotypically characterise the acquisition of vancomycin resistance in *C. difficile*, through large-scale experimental evolution and genome sequencing. A broth-based gradient evolution was performed using 10 independent engineered clinically-relevant R20291 lines, over approximately 250 generations. End-point isolates and whole populations were sequenced, and a custom bioinformatics pipeline was developed to identify contributing mutations. Vancomycin resistance (up to 32x MIC) evolved rapidly across all 10 lines, however this was accompanied by significant fitness defects in both growth and sporulation. Population data revealed complex evolutionary dynamics, and striking evidence of parallel evolution, as well as enabling identification of multiple genes associated with vancomycin resistance. More broadly, multiple routes to resistance were identified, including a *vanTG*-mediated pathway, present in 4/10 independent lines; and a pathway involving a previously-uncharacterised two-component system, present in 6/10 independent lines. This work provides the starting point of a detailed understanding of vancomycin resistance in this important pathogen, which in future may inform clinical surveillance strategies.

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A single-cell resolution, multi-omic spatial atlas of colonic tumorigenesis driven by *C. difficile* from human colorectal cancer-associated biofilms

Emily Green[1], Megan Rutheford[2], Subhag Kotrannavar[1], Logan Vlach[2], Hannah Lunnemann[1], Cody Heiser[1], Shaoguang Wu[3], Hua Ding[3], J. Alan Simmons[1], Xiao Liu[4], Martha Shrubsole[2], Qi Liu[4], Ken Lau[1], D. Borden Lacy[5], Cynthia Sears[3], Robert Coffey[1], Julia Drewes[3], Nicholas Markham[6]

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Emerging evidence strongly supports a causal role for specific pro-carcinogenic driver bacteria within the colonic microbiota. Invasive bacterial biofilms may initiate or accelerate colorectal cancer (CRC) through epithelium-autonomous or inflammation-dependent mechanisms. To better understand host-microbe interactions during colonic tumorigenesis, we combined single-cell RNA-sequencing (scRNA-seq), spatial transcriptomics, and immunofluorescence to define the molecular spatial organization of colonic tissue from germ-free *Apc*$^{Min/+}$ mice colonized with bacteria from human biofilm-associated CRC. We previously demonstrated toxigenic *C. difficile* is a critical species within human CRC biofilms for driving adenoma formation in this mouse model. Here, we focus on comparing germ-free *Apc*$^{Min/+}$ mice gavaged with biofilm-positive human CRC-derived bacteria either with or without *C. difficile*. To investigate mechanisms of tumorigenesis, tissue was harvested two weeks post-inoculation for scRNA-seq and spatial transcriptomics. After routine processing of the scRNA-seq dataset, we applied Markov state modeling to infer differentiation cell states. We illustrated random walks that suggest terminal differentiation in a cell cluster that is simultaneously enriched with G2/M- and S-phase cell cycle genes. In absorptive colonocytes, our differential gene expression analysis showed the gastric metaplasia-associated glycoprotein *Dmbt1* is up-regulated by *C. difficile*. Surprisingly, our spatial transcriptomic analysis showed *Dmbt1* was dramatically down-regulated in dysplastic foci compared with normal-appearing tissue. We are currently integrating these datasets and mechanistically testing how Dmbt1 affects tumorigenesis. In summary, we present data from a human mucosal biofilm-associated colonic tumorigenesis model at single-cell resolution to reveal novel cell type-specific transition states and signaling patterns during bacteria-driven tumorigenesis.

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Recent developments in the treatment and prevention of *C. difficile* infection

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Larry Kociolek, MD MSCI is an Attending Physician in the Division of Infectious Diseases and Associate Medical Director of Infection Prevention and Control at Ann & Robert H. Lurie Children's Hospital of Chicago. He joined the Lurie Children's faculty in 2014 and is an Assistant Professor of Pediatrics at Northwestern University Feinberg School of Medicine. Dr. Kociolek's research efforts are focused on reducing infections that commonly develop in hospitals and other healthcare environments, particularly C*. difficile* infection. He is Principal Investigator of two active NIH-funded translational research studies. Dr. Kociolek is an active member of the Society for Healthcare Epidemiology of America (SHEA). He serves on the SHEA Pediatric Leadership Council Steering Committee and is leading a writing group to define best practices for CDI prevention. Dr. Kociolek serves as an Associate Editor of the *Journal of the Pediatric Infectious Diseases Society*.

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Functional microbiome variation influences *Clostridioides difficile* infection susceptibility

Anna DeVeaux[1, 2], Skye Fishbein[1, 2], Aura Ferreiro[1, 2, 3], Tiffany Hink[4], Kimberley Reske[4], Erik Dubberke[4], Gautam Dantas[1, 2, 3, 5, 6]

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Clostridioides difficile infection (CDI) is a leading cause of healthcare-associated diarrhea and community infection is increasingly reported. CDI development is complex, underscored by the following insights: 1) not all patients colonized with *C. difficile* develop CDI after antibiotic exposure, 2) not all CDI patients have a recent antibiotic exposure history, and 3) specific gut commensals have increasingly been associated with CDI risk. We sought to understand how functional variation in the gut microbiome of susceptible patients influences CDI susceptibility. We analyzed stool metagenomes of 63 CDI patients (enzyme immunoassay (EIA)+) and 80 *C. difficile*-colonized patients without CDI (EIA-) at Barnes-Jewish Hospital in St. Louis, MO. Compared to EIA- patients, EIA+ patients had significantly lower butyrogenic taxa, including *Dorea* (q = 0.0049), and significantly higher *Fusobacterium* and *Enterococcus* (q = 0.09), as determined by GLMMs. To investigate microbiome constituents associated with protection from CDI independent of antibiotic treatment, we engrafted 16 microbiomes from EIA- patients into gnotobiotic mice (4-8 mice per arm) and monitored animal health for 21 days. 12 donor microbiomes resulted in >50% survival and represented a spectrum of stable *C. difficile*-microbiome interactions; these groups clustered by changes in *C. difficile* CFUs over time (Pearson correlation, R = 0.7, $p = 1.5e^{-8}$). *Ruminococcus gnavus* was significantly associated with an increase in *C. difficile* (q = 0.009), whereas *Bacteroides ovatus* was associated with decreasing *C. difficile* levels (q = 0.0005) by GLMMs. We predict that these species participate in ecological networks that alter the niche availability for *C. difficile*.

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Clostridioides difficile PCR ribotype 151 is polyphyletic and includes pathogenic isolates from cryptic clade C-II with mono-toxin pathogenicity loci that can escape routine diagnostics

Quinten R. Ducarmon[1], Jan-Tom van der Bruggen[2], Céline Harmanus[1], Ingrid M.J.G. Sanders[1], Laura G.M. Daenen[2], Ad C. Fluit[2], Rolf H.A.M. Vossen[1], Susan L. Kloet[1], Ed J. Kuijper[1], Wiep Klaas Smits[1]

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We report a patient case with pseudomembranous colitis associated with a mono-toxin producing *Clostridioides difficile* belonging to the very rarely diagnosed PCR ribotype (RT) 151. The infection was difficult to diagnose, since the isolate and the feces sample tested negative for toxin-encoding genes using a routine commercial test.

This prompted us to sequence n = 11 RT151s from various geographical regions to study their genomic characteristics and relatedness. By including whole genome sequence data from other sources, we could further place these isolates into the phylogenetic tree of *C. difficile* and assign them to their respective clades.

These analyses revealed that 1) RT151s are polyphyletic with isolates falling into clades 1, and cryptic clades C- I and C- II 2) RT151 contains both non-toxigenic and toxigenic isolates and 3) RT151 C-II isolates contained mono-toxin pathogenicity loci (PaLoc) (1). Additional analysis with PacBio circular consensus sequencing revealed that the isolate from our patient case report contains a novel PaLoc insertion site, lacked *tcdA* and a had significantly divergent *tcdB* sequence that might explain the failure of the diagnostic test. We also suggest that certain isolates in literature may have been misclassified and also belong to cryptic clade C-II (2,3).

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2. Janezic *et al*. J Clin Microbiol. 2015; 53(2):692-5. doi: 10.1128/JCM.02211-14.
3. Monot *et al*. Sci Rep. 2015; 5:15023. doi: 10.1038/srep15023.

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A dual-action antibiotic that kills *Clostridioides difficile* vegetative cells and inhibits spore germination

Jeshina Janardhanan[1], Choon Kim[1], Yuanyuan Qian[1], Jingdong Yang[1], Jayda Meisel[1], Derong Ding[1], Enrico Spei[1], Valerie Schroeder[1], William Wolter[1], Allen Oliver[1], Shahriar Mobashery[1], Mayland Chang[1]

[1]University of Notre Dame

Clostridioides difficile infection (CDI) is the most lethal of the five CDC urgent publichealth treats, resulting in 12,800 annual deaths in the United States alone [Antibiotic Resistance Threats in the United States, 2019 (2019), www.cdc.gov/DrugResistance/Biggest-Threats.html].The high recurrence rate and the inability of antibiotics to treat such infections mandate discovery of new therapeutics. A major challenge with CDI is the production of spores, leading to multiple recurrences of infection in 25% of patients [C. P. Kelly, J. T. LaMont,N. Engl. J. Med. 359, 1932-1940 (2008)], with potentially lethal consequence. Herein, we describe the discovery of an oxadiazole as a bactericidal anti-C. difficile agent that inhibits both cell-wall peptidoglycan biosynthesis and spore germination. We document that the oxadiazole binds to the lytic transglycosylase SleC and the pseudoprotease CspC fo rprevention of spore germination. SleC degrades the cortex peptidoglycan, a critical step in the initiation of spore germination. CspC senses germinants and cogerminants. Binding to SleC is with higher affinity than that to CspC. Prevention of spore germination breaks the nefarious cycles of CDI recurrence in the face of the antibiotic challenge, which is a primary cause of therapeutic failure. The oxadiazole exhibits efficacy in a mouse model of recurrent CDI and holds promise in clinical treatment of CDI.

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Alarmone Signaling in *C. difficile*: Newly discovered variations on a conserved pathway

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Erin Purcell earned her Ph.D. in Biochemistry and Molecular Biophysics at the University of Chicago before completing her post-doctoral research at the University of North Carolina at Chapel Hill. In 2016 she established her lab at Old Dominion University studying stress survival and alarmone signaling in *Clostridioides difficile* and other Gram-positive pathogens.

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HRMAS 13C NMR and genome-scale metabolic modeling identify threonine as a preferred dual redox substrate for *Clostridioides difficile*

Aidan Pavao[1], Ella Zhang[2], Auriane Monestier[3], Johann Peltier[4], Bruno Dupuy[3], Leo Cheng[2], Lynn Bry[1]

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Stickland fermenting *Clostridia* can employ sole metabolism of amino acids to rapidly produce energy and anabolic substrates for growth. These species prefer dual redox substrates, including ones available in gut ecosystems such as mucin-abundant leucine. Here, we demonstrate theronine's role as a dual redox substrate for the pathogen *Clostridioides difficile*. High-Resolution Magic Angle Spinning (HRMAS) NMR spectroscopy, with dynamic flux balance analyses (dFBA), showed sequential recruitment of four distinct threonine fermentation pathways to support changing needs for energy production, redox balance, biomass, and nitrogen cycling. Model predictions and ^{13}C isoptomer analyses of [U-^{13}C]threonine-origin metabolites inferred reductive metabolism of threonine through the reductive leucine pathway, a finding confirmed by deletion of the *hadA* 2-hydroxy-isocaproate CoA transferase. *In vivo* metabolomic and metatranscriptomic analyses in mice mono- or co-colonized with the protective commensal *Paraclostridium bifermentans* or *C. difficile,* demonstrated active depletion of gut pools of threonine, with differential expression of theonine fermentation systems in each organism over the course of infection. Our findings expand the range of *C. difficile* preferred *in vivo* substrates and metabolic targets to limit pathogen colonization while enhancing Stickland metabolism in protective commensal species.

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Iron storage organelles facilitate *Clostridioides difficile* survival in the gut

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Iron is indispensable for almost all forms of life but toxic at elevated levels. To survive within their hosts, bacterial pathogens have evolved iron uptake, storage, and detoxification strategies to maintain iron homeostasis. However, these iron homeostatic systems are largely undefined in the human pathogen *Clostridioides difficile*. Here, we report that *C. difficile* undergoes an intracellular iron biomineralization process and stores iron in membrane-bound ferrosome organelles containing non-crystalline iron phosphate biominerals. We found that a membrane protein (FezA) and a P_{1B6}-ATPase transporter (FezB), repressed by both iron and the ferric uptake regulator Fur, are required for ferrosome formation and play an important role in iron homeostasis during transition from iron deficiency to excess. Additionally, using two mouse models of *C. difficile* infection (CDI), we demonstrated that the ferrosome system is activated in the inflamed gut to combat calprotectin-mediated iron sequestration and is important for bacterial colonization and survival during CDI. Further studies are underway to elucidate the molecular basis of ferrosome biogenesis. Nonetheless, the ferrosome nanoparticles have the potential to redefine the concept of trace element storage in anaerobes, unveil important insight into how gut microbes cope with changes in elemental levels within the hosts, and provide a prototype for production of metal nanoparticles and drug delivery vesicles, and other bionanotechnology applications. Finally, the importance of ferrosomes to combat nutritional immunity establishes ferrosome formation as a new target for the development of antimicrobial therapeutics against this important pathogen.

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Contact of *Clostridium perfringens* type A strain ATCC3624 with C2C12 muscle cells increases toxin production to enhance ATCC3624 growth in a process involving the EutV/W two component system

Jihong Li[1], Bruce McClane[1]

[1]University of Pittsburgh

Clostridium perfringens type A causes gas gangrene (clostridial myonecrosis), which involves muscle infection. Previous studies by the Rood laboratory showed that alpha toxin (PLC) and perfringolysin O (PFO) are important for type A strains to cause gas gangrene in a mouse model. One possible contribution of these toxins to pathogenesis could involve generating growth nutrients from toxin-damaged muscle cells. This hypothesis was tested by comparing the growth of *C. perfringens* type A strain ATCC3624 and its toxin null mutants or complementing strains in an *in vitro* model using differentiated C2C12 muscle cells as the only nutrient source. Results showed both PFO and PLC support ATCC3624 growth using C2C12 cells. Both toxins were cytotoxic, suggesting their growth support in this model involves releasing nutrients from C2C12 cells. Moreover, both toxins worked together synergistically to cause C2C12 cell cytotoxicity and support ATCC3624 growth. qRT-PCR analyses showed contact of ATCC3624 with C2C12 cells induces rapid upregulation of *plc* and *pfoA* expression, as well as increased expression of *virS/virR* and *agrB/D,* which regulate expression of these toxins. Additionally, ATCC3624 contact with C2C12 cells also increased expression of the *eutV/W* (which regulates expression of genes involved in ethanolamine utilization) two component-regulatory system and a *eutV/W* null mutant showed impaired growth using C2C12 cells. These results suggest a model where, during gas gangrene, increased PFO and PLC damage muscle cells to release ethanolamine, which is then used for growth.

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Reengineering of the gut bile acid landscape to restrict *C. difficile*

Casey Theriot[1]

[1]North Carolina State University

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Casey M. Theriot, PhD, is an Associate Professor in Infectious Disease at North Carolina State University, College of Veterinary Medicine in Raleigh, NC. She received a BES in Environmental Science from the University of Georgia, and her PhD in Microbiology from North Carolina State University. She has also worked at the Centers for Disease Control and Prevention (CDC) as a Microbiologist. She completed a postdoctoral fellowship and independent research position with Dr. Vincent Young at the University of Michigan Medical School, where she focused on defining the gut microbiome and metabolome during resistance and susceptibility to *Clostridioides difficile* colonization and infection in a mouse model. Dr. Theriot's current research focuses on defining how the gut microbiota is able to provide colonization resistance against pathogens including *C. difficile*. Two mechanisms that she continues to focus on are 1) the role of gut microbial derived secondary bile acids and their ability to inhibit *C. difficile,* and 2) competition of nutrients by members of the microbiota, specifically members from the commensal *Clostridia.* She is also working on manipulating the gut microbiota to rationally alter the composition of the bile acid and amino acid pool in the gut, which has the potential to improve preventative and therapeutic approaches against *C. difficile* infection and many other human diseases. The goal of her work is to design targeted bacterial approaches to prevent and treat gastrointestinal diseases – improving clinical outcomes. Dr. Theriot is a member of the Comparative Medicine Institute at NCSU and the Center for Gastrointestinal Biology and Disease at UNC. She has received multiple pilot and NIH research awards for her research on *C. difficile* including a Mentored Research Scientist Development Award in Metabolomics (K01), and the Maximizing Investigators' Research Award (MIRA) (R35) from the NIGMS.

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A solid movement with RBX2660. Safely restoring gut microbiota from donor to patient

Thomas Louie[1]

[1]Ferring Pharmaceuticals / University of Calgary

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Control of *C. difficile* toxins and repair of the gut endothelium by a microbial metabolite

Venkatakrishna Jala[1], Sweta Ghosh[1], Michelle Chua[1], Daniel Erickson[1], James Collins[1]

[1]University of Louisville

Toxins produced by *C. difficile* (TcdA and TcdB) cause intestinal epithelial injury and lead to severe gut barrier dysfunction, stem cell damage, and impaired regeneration. Preliminary findings indicate that treatment with the microbial metabolite urolithin A (UroA) can attenuate CDI-induced adverse effects on the colon epithelium in a mouse model and protect intestinal tight junction proteins from disruption. We also observed that UroA treatment enhanced gut barrier function by upregulating tight junction proteins and mucin levels and increasing intestinal stem cell proliferation. Furthermore, biologically relevant concentrations of UroA reduced toxin production *in vitro*, without affecting the growth rate or survival of *C. difficile*, in a dose-dependent manner. Based on these observations, we hypothesize that UroA protects against CDI by reducing *C. difficile* toxin production and protects against toxin-induced gut barrier dysfunction by restoring tight junction proteins and stem cell functions, leading to the mitigation of colitis.

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Dr. Tohru Shimizu Memorial Lecture:

With a little help from my friends: *Clostridium perfringens* Quorum Sensing

Bruce McClane[1]

[1]University of Pittsburgh

Bruce McClane received his PhD from Penn State and performed post-doctoral research at NYU School of Medicine. He has worked on *Clostridium perfringens* since 1976, focusing on such topics as toxin mechanism of action, pathogenesis, contributions of plasmids to virulence, and gene regulation. Dr. McClane has published over 200 papers on these topics and his research has been continuously funded by NIH since 1982, including 10 years as a MERIT award. He is currently an Editor of *PLoS Pathogens*, *mBio* and *Toxins*; he also serves on the editorial boards of *Infection and Immunity* and *Anaerobe*. Dr. McClane is a fellow of the American Academy of Microiology and a founder of the International Conference on the Molecular Biology and Pathogenesis of the Clostridia (Clostpath).

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Non-antibiotic strategies to mitigate *C. difficile* infection: insights from discovery research

Dena Lyras[1] [1]Monash University

Professor Dena Lyras is the Deputy Director of the Biomedicine Discovery Institute at Monash University. Her laboratory is focused on enteric pathogens, particularly the clostridia and those involved in antibiotic-associated diarrhea in humans and animals, and they use genetic approaches to understand how these microbes harness regulatory and virulence factors to interact with the host and cause disease. Antibiotic resistance and DNA mobility are also research areas of focus, in the context of gut pathogens and antibiotic-associated diarrheal disease. In collaboration with industry and academic partners, her laboratory is developing immunotherapeutics and small molecules to prevent and treat these infections. She was awarded an Australian Research Council Future Fellowship in 2012 and began an Australian Research Council Laureate Fellowship in 2022.

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Presynaptic targeting of botulinum neurotoxin type A requires a tripartite PSG-Syt1-SV2 plasma membrane nanocluster for synaptic vesicle entry

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The unique nerve terminal targeting of botulinum neurotoxin type A (BoNT/A) is due to its capacity to bind two receptors on the neuronal plasma membrane: polysialoganglioside (PSG) and synaptic vesicle glycoprotein 2 (SV2). Whether and how PSGs and SV2 may coordinate other proteins for BoNT/A recruitment and internalization remains unknown. Here, we demonstrate that the targeted endocytosis of BoNT/A into synaptic vesicles (SVs) requires a tripartite surface nanocluster. Live-cell super-resolution imaging and electron microscopy of catalytically inactivated BoNT/A wildtype and receptor-binding-deficient mutants in cultured hippocampal neurons demonstrated that BoNT/A must bind coincidentally to a PSG and SV2 to target synaptic vesicles. We reveal that BoNT/A simultaneously interacts with a preassembled PSG-synaptotagmin-1 (Syt1) complex and SV2 on the neuronal plasma membrane, facilitating Syt1-SV2 nanoclustering that controls endocytic sorting of the toxin into synaptic vesicles. Syt1 CRISPRi knockdown suppressed BoNT/A- and BoNT/E-induced neurointoxication as quantified by SNAP-25 cleavage, suggesting that this tripartite nanocluster may be a unifying entry point for selected botulinum neurotoxins that hijack this for synaptic vesicle targeting.

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Holin-dependent secretion of the large clostridial toxin TpeL by *Clostridium perfringens*

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All Clostridial pathogens secrete toxins as part of their pathogenesis. One family of toxins produced are the large clostridial cytotoxins (LCTs), which include TpeL from *C. perfringens,* TcdA and TcdB from *C. difficile*, and TcsL and TcsH from *P. sordellii*. LCTs glycosylate small G-proteins in mammalian host cells, disrupting cell signaling, leading to cell death. While these toxins all lack a signal peptide for Sec-dependent secretion, the pathogenicity locus (PaLoc) encoding each toxin gene also encodes a phage holin-like protein, TpeE for *C. perfringens*, TcdE for *C. difficile*, and TcsE for *P. sordellii*. Each toxin has been shown to be dependent on their cognate holins for secretion. While TpeE shares membrane topology with the TatA protein, which is involved in the Twin-Arginine Transport (Tat) secretion system, TcdE and TcsE share membrane topology with *E. coli* phage lambda holin S^{105}. We have developed a model for TpeE-dependent secretion in which TpeL partially inserts into the cytoplasmic membrane, which causes TpeE to oligomerize and form a channel for TpeL secretion. We have developed novel technologies to analyze this model including protein cross-linking, fluorescent protein fusions and time-lapse imaging and our results provide support for our model thus far. Since TpeE has the same membrane topology as the pore-forming TatA protein, we hypothesize that this model also applies to secretion by the Tat system. Because there are >1,000 homologs of TpeE holin-like proteins found in Gram-positive bacteria, there are likely many other still to be discovered secretion systems that utilize this mechanism.

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Understanding surface variability in *Clostridioides difficile* spores and implications in disease.

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Dr. Marjorie Pizarro-Guajardo earned her Ph.D. in 2018 under the supervision of Dr. Daniel Paredes-Sabja at the Universidad Andres Bello, Chile, investigating the variability of *C. difficile* spore surface. She is currently an Assistant Research Scientist at Texas A&M University. Her research focuses understand how *C. difficile* assembles spores that have two exosporium morphotypes (thick and thick-exosporium layers) and the role of both exosporium-morphotypes and the hair-like projections of the spore surface in the pathogenesis of the infection. Dr. Pizarro-Guajardo is also working on the development of a vaccine prototype to prevent *C. difficile* spore-persistence.

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Spore Ultrastructure and Germination in *Clostridium sporogenes*

Hannah Fisher[1], Robert Fagan[1], Per Bullough[1]

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Clostridium botulinum produces the most potent neurotoxin known to man, poses a significant challenge to the food industry and is considered a bioterrorism threat. *Clostridium sporogenes* is a widely used, genetically similar, surrogate for the study of Group I *C. botulinum*. The production of highly resilient dormant spores and their subsequent germination is important for transmission and survival of these species, particularly through aerobic environments.

Spore germination is triggered by germinant molecules in the environment resulting in the transition from highly resistant, dormant spore to metabolically active vegetative cell. Despite being of significant importance, the process of germination and subsequent outgrowth in *C. sporogenes* is relatively poorly understood both morphologically and biochemically. Of particular interest is how the emerging vegetative cell breaks through the highly resilient paracrystalline exosporium that makes up the outermost layer of the spore.

Using a range of microscopy techniques, it is possible to probe spore ultrastructure and track the morphological changes occurring throughout the germination process. We have conducted live cell imaging of *C. sporogenes* spore germination showing clear forceful polar emergence of the vegetative cell. Germination has been further characterised by transmission electron microscopy (TEM) of germinating spores. Producing knockouts of key exosporium associated proteins has allowed us to delve deeper into the exosporium structure and its role in germination. We will report on the roles of these exosporium associated proteins and of the overall molecular architecture of the spore in guiding the emerging vegetative cell into the environment.

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C. difficile engulfasome at the molecular level

Anna Barwinska-Sendra[1], Charmaine Normington[2], Marcin Dembek[1], Charlotte G. Roughton[1], Victoria Burge[1], Abigail Kelly[1], Melanie L. Hutton[3], Caitlin Jukes[4], Daniella Vollmer[1], Joe Gray[1], Mark Wilcox[2], Waldemar Vollmer[1], Dena Lyras[3], Gillian G. Douce[4], Anthony Buckley[2, 5], Paula S. Salgado[1]

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C. difficile spores are the infective agent of *C. difficile* infections. Sporulation is a complex differentiation programme, initiated by the master regulator Spo0A. It begins with asymmetric cell division, followed by engulfment of the forespore by the mother cell.

Studies in *B. subtilis* and *C. difficile* have so far been identified and characterised two components of the engulfasome: the peptidoglycan degradation machinery formed by SpoIID, SpoIIM and SpoIIP and the complex formed by the mother cell SpoIIIAH and the forespore SpoIIQ proteins (Q:AH).

We previously showed that, in *C. difficile*, Q:AH and D/P proteins are essential for engulfment whilst, surprisingly, SpoIIM, is not required. Q:AH play a role in gene expression during sporulation and D/P sequential peptidoglycan degradation activity allows engulfment of the forespore.

Recently, we have investigated the role of Q:AH in virulence to assess its suitability as a potential therapeutic targets. Unlike mutants of Spo0A, that can exhibit increased virulence, *spoIIQ* and *spoIIIAH* mutants do not have a detrimental disease effect. Importantly, disrupting Q:AH halts persistence in vivo and affects biofilms, showing this is a potential route to prevent recurrence and interrupt the infection cycle.

We aim to define ligand specificity of SpoIIP and SpoIID and activity mechanisms at the atomic level. We have also established methods to identify other engulfasome proteins, combining of biophysical, biochemical and enzymatic assays, coupled with state-of-the-art 'omics approaches.

Understanding the molecular details of the engulfasome and is role in pathogenicity are essential to develop more targeted, novel therapeutic approaches to tackling CDI.

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Pseudoprotease-mediated regulation of germinant sensing in *Clostridioides difficile*

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Clostridioides difficile infection begins when metabolically dormant spores encounter germinants in the vertebrate gut and initiate the process of germination. This process is essential for *C. difficile* to produce vegetative cells that release toxins and cause disease. *C. difficile* germination differs from that of most spore-forming bacteria. First, *C. difficile* spores germinate in response to bile acid germinants combined with amino acid and/or Ca^{2+} co-germinants. Second, while most spore-formers sense germinants through highly conserved transmembrane receptors, *C. difficile* does not encode these receptors and instead uses soluble pseudoproteases (CspA and CspC) to sense co-germinants and germinants, respectively. CspA and CspC then go on to activate CspB, which activates a lytic enzyme responsible for degrading the protective cortex layer of the spore. While CspA and CspC clearly play key roles in regulating germination initiation, the mechanism by which they integrate (co-)germinant signals is poorly understood. We recently discovered that CspC and CspA form a heterodimer, while CspA forms a homodimer. By solving the crystal structure of the CspA homodimer, we identified residues that regulate CspA homodimerization. Mutation of these CspA homodimerization residues promotes CspA:CspC heterodimerization *in vitro* and reduces the sensitivity of *C. difficile* spores to bile acid germinant. These biochemical and genetic analyses suggest that CspC and CspA integrate (co-)germinant signals using a “Partner-Swap” model in which (co-)germinants encountered in the gut destabilize the CspC:CspA heterodimer and promote CspA homodimerization. Liberated CspC may then modulate activity of CspB, allowing for subsequent germination of the spore.

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Identification of a Two-Component Signal Transduction System That Responds to Lipid II-Interacting Antibiotics

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Craig Ellermeier is a Professor of Microbiology and Immunology at the University of Iowa. His research focuses on cell envelope stress response systems in Gram positive bacteria including *C. difficile*, *B. subtilis*, and *B. thuringiensis*. His work has resulted in the dissection of signal transduction systems that respond to host innate immune factors like lysozyme as well as identification of signal transduction systems required for resistance to antibiotics that target the cell envelope of *C. difficile*.

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Identification of orphan histidine kinases that impact sporulation and enterotoxin production by *Clostridium perfringens* type F strain SM101 in a pathophysiologically-relevant *ex vivo* mouse intestinal contents model

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To cause gastrointestinal disease, *Clostridium perfringens* type F strains must sporulate to produce *C. perfringens* enterotoxin (CPE) in the intestines. *C. perfringens* is thought to use some of its seven annotated orphan histidine kinases to phosphorylate Spo0A and initiate sporulation and CPE production. We previously demonstrated the CPR0195 orphan kinase (OK), but not the CPR1055 OK, is important for type F strain SM101 to sporulate and produce CPE in MDS sporulation medium. In the absence of an animal model for *C. perfringens* sporulation, the current study used diluted mouse intestinal contents (MIC) to develop an *ex vivo* sporulation model and employed this model to test sporulation and CPE production by SM101 CPR0195 and CPR1055 null mutants in a pathophysiologically-relevant context. Surprisingly, both mutants still sporulated and produced CPE at wild-type levels in MIC. Therefore, five single null mutants were constructed that cannot produce one of the previously-unstudied SM101 OKs. Those mutants implicated CPR1316, CPR1493, CPR1953 and CPR1954 in sporulation and CPE production by SM101 MDS cultures; it was then determined that phosphorylation activity is necessary for these effects, confirming the identity of these four proteins as kinases. Importantly, only the CPR1953 or CPR1954 null mutants exhibited significantly reduced sporulation and CPE production levels in MIC cultures. Characterization studies suggested that, in MDS or MIC, the CPR1953 and CPR1954 mutants produce less Spo0A than wild-type SM101; the CPR1954 mutant also exhibited little/no Spo0A phosphorylation in MDS cultures. These findings link CPR1953 and CPR1954 to *C. perfringens* sporulation/CPE production in disease-relevant conditions.

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The armada of oxidative stress detoxication enzymes of *C. difficile*

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Clostridioides difficile is a “strict” anaerobe. However, in the gastro-intestinal tract, it is exposed to low oxygen (O_2) tensions around 0.1-0.4% in the lumen and 1% near the epithelium of the colon and 4-5% in the upper intestine, where spores germinate. It is also exposed to ROS produced endogenously or exogenously by the immune system.

An armada of proteins involved in oxidative stress detoxication has been identified in *C. difficile*. We showed that the 4 O_2-reductases, have different spectra of activity depending on the O_2 tensions, with both revRbr being more specific of low O_2, FdpF of high O_2 and air and FdpA having a more intermediate role. These differential spectra of activity seem to be associated with differences in gene regulation rather than actual differences in enzymatic activity.

Our results also suggest the existence of an unidentified 5th O_2-reductase. We characterized enzymatically the Rbr1 and showed that it is the remaining O_2-reductase, but also the main peroxidase of our strain. Moreover, the *rbr1* gene is found in an operon specific of ROS detoxication, which also encodes a predicted superoxide reductase (Sor) and PerR, a regulator responsive to H_2O_2. We characterized enzymatically the Sor and showed that Rbr1, PerR and Sor are all involved in the tolerance to ROS, high O_2 tensions and air, but not to lower O_2 tensions. This suggests that high O_2 tensions and air are complex stresses encompassing both the presence of O_2 and an important endogenous ROS production.

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The RgaSR two-component system promotes *Clostridioides difficile* sporulation through a small regulatory RNA and the AgrD1 autoinducing peptide

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Bacterial endospore formation is initiated by phosphorylation of the transcriptional regulator, Spo0A. Multiple kinases activate Spo0A in other spore-forming organisms; however, these factors are not conserved in *Clostridioides difficile*. Three phosphotransfer proteins modulate *C. difficile* sporulation, but phosphorylation of Spo0A in *C. difficile* is poorly understood. Here, we searched for additional factors that may phosphorylate Spo0A. We found that deletion of an orphan histidine kinase, RgaS, significantly reduced *C. difficile* sporulation frequency. RgaS shares homology with AgrC and VirS, conserved histidine kinases of accessory gene regulator (Agr) quorum-sensing systems. The majority of *C. difficile* genomes encode a partial *agr* locus, comprised of the autoinducing peptide (*agrD1*) and a protease (*agrB1*), but a cognate AgrC histidine kinase and AgrA response regulator remain unidentified. Previous work showed that the *agrB1D1* locus positively influences early stage sporulation. Additionally, a VirR-like orphan response regulator, RgaR, was shown to directly activate *agrB1D1* transcription. We found that deletion of *rgaR* resulted in significantly decreased sporulation, comparable to the *rgaS* mutant. Transcript levels of direct RgaR targets, including *agrB1D1*, were similarly decreased in the *rgaS* and *rgaR* mutants, suggesting that RgaS and RgaR function as a cognate two-component system. We discovered that a direct RgaR target, the small regulatory RNA *srsR*, positively impacts later stages of sporulation. Unlike other Agr systems, we observed that the RgaSR two-component system was not was not activated by the AgrD1 peptide. These data reveal that RgaSR promotes different stages of *C. difficile* sporulation through two independent regulatory pathways.

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Clostridioides difficile nucleobase scavenging in the competitive gut environment

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Clostridioides difficile is the leading cause of nosocomial infectious diarrhea, and faces competition for nutrients from both the microbiota and immune system during infection. Amongst these nutrients are nucleobases, and nucleobase metabolism is critical for pathogenesis. We found that *C. difficile* can utilize 4-thiouracil (4-TU) as a uracil source in the vertebrate gut. The metabolism of 4-TU is mediated by proteins containing domain of unknown function 523 (DUF523). *C. difficile* encodes for two DUF523 paralogs, one of which is required for growth in the presence of 4-TU and which we have named, TudS. Additionally, *Escherichia coli* lacks a DUF523 homolog and 4-TU inhibits growth. We found that heterologous expression of *C. difficile tudS* is sufficient to restore growth of *E. coli* in 4-TU. Due to the structural similarity between 4-TU and uracil, we hypothesized that 4-TU is toxic because of misincorporation into RNA. Indeed, we discovered that 4-TU is incorporated into RNA in the absence of TudS. To identify additional components involved in 4-TU metabolism, we conducted a genetic selection in *E. coli* in the presence of 4-TU. We discovered that mutations in uracil phosphoribosyltransferase, a component of the uracil salvage pathway, are sufficient to overcome 4-TU toxicity and prevent 4-TU incorporation into RNA. These data suggest that 4-TU hijacks the uracil salvage pathway, and *C. difficile* exploits this through TudS conversion of 4-TU to uracil in the first step of 4-TU salvage. We hypothesize that this metabolic mechanism gives *C. difficile* an advantage in the competitive gut environment.

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Of mice and women: the effect of the estrous cycle on *Clostridioides difficile* infection outcomes

Jacqueline Phan[1], McKenzie Washington[1], Dung Do[1], Tiffany Mata[1], Maria Niamba[1], Efren Heredia[1], Robert Soriano[1], Chandler Hassan[1], Chad Cross[1], Ernesto Abel-Santos[1]

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Clostridioides difficile infection (CDI) is responsible for the majority of identifiable hospital-related antibiotic-associated diarrhea. Susceptibility to CDI and severity of disease varies depending on a variety of factors such as aggressive use of broad-spectrum antibiotics, age, and immune status. Epidemiological studies have consistently shown that female patients are more at risk for CDI than their male counterparts. In this study, we show that female mice developed more severe CDI than their male counterparts when challenged with spores from three different *C. difficile* strains. CDI sexual dimorphism was still apparent when animals were placed under diet conditions that exacerbated CDI severity. To study the effect of estrous cycle on CDI, female mice were challenged with *C. difficile* spores when they were at the estrus, metestrus, diestrus, late diestrus/early proestrus, proestrus, or late proestrus/early estrus stages. Animals were scored for CDI severity while monitoring their estrous cycle stages. The resulting data showed a striking spike in CDI severity when animals were in proestrus the day before. In contrast, animals who were in estrus the prior day were protected from CDI. Prophylactic treatment of CDI also showed sexual dimorphism with females responding better to treatmentthan males. Interestingly, infection sexual dimorphism was reversed in hamsters, with male hamsters developing more severe CDI signs than females. In conclusion, we have shown that mice recreate many of the conditions of sexual dimorphism of human CDI.

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Structural characterization of bile acid inhibition of *Clostridioides difficile* Toxin B

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Intestinal bile acids have been shown to play key roles in the life cycle of *Clostridioides difficile* by either promoting spore germination or inhibiting growth and virulence. Recently, we discovered that intestinal bile acids are also potent inhibitors Toxin B (TcdB), the primary virulence determinant of *C. difficile*. The molecular mechanisms by which these small molecules inhibit the function of the large 270-kDa TcdB toxin, however, are unclear. In this work, we present the first cryoEM structure of TcdB bound to a small molecule inhibitor, the secondary bile acid derivative methyl cholate. We describe how this molecule is binding to the toxin and validate the binding interface. Furthermore, this structure presents a unique conformation of TcdB which we interpret to propose a structural mechanism for bile acid inhibition of TcdB. Taken together, our work provides insight into the complex role bile acids play in *C. difficile* virulence and could be used to design novel therapeutics to treat infection.

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C. difficile TcdB-receptor tropism and binding interactions are driven by extracellular calcium

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Disease manifestation of the gastrointestinal pathogen *Clostridioides difficile* is driven by the intoxication of host cells by the exotoxin TcdB. Among the receptors utilized for cell entry, the ability to bind chondroitin sulfate proteoglycan 4 (CSPG4) is evolutionarily conserved between the two major variants of TcdB, TcdB1 and TcdB2. Interestingly, CSPG4 does not typically undergo receptor-mediated endocytosis but instead, is cleaved from the cell surface to function as a potent signaling molecule. This led us to investigate specific environmental factors that stabilize interactions between TcdB and CSPG4 to promote cell binding and entry. Changes in luminal calcium have been shown to play important roles in *C. difficile* pathogenesis. Yet, whether calcium serves a role in driving toxin-receptor interactions remained unknown. Using a series of TcdB receptor binding mutants and cell lines with various receptor expression profiles, we discovered calcium regulates TcdB interactions with host cells in a receptor-dependent manner. Specifically, TcdB preferentially binds CSPG4 under increased concentrations of calcium, while the absence of calcium promotes CSPG4-independent cell entry. Moreover, using a combination of biochemical and biophysical techniques we determined calcium drives interactions between TcdB and chondroitin sulfate - the sole glycosaminoglycan of CSPG4. As the chondroitin sulfate chains of CSPG4 are known to regulate surface cleavage, we propose interactions between the chondroitin sulfate chains and TcdB could potentially disrupt CSPG4 cleavage to promote toxin uptake. These data suggest changes to the colonic microenvironment, such as the availability of divalent cations, could have substantial impacts on TcdB's ability to promote disease.

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Understanding the role of a key sporulation specific protein in beta-lactam resistance in *Clostridioides difficile*

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Clostridioides difficile, a spore forming bacterium, is a leading cause of nosocomial infections. *C. difficile* infections can cause mild symptoms such as diarrhoea, through to more severe disease symptoms, including inflammation and enlargement of the colon. Spores are crucial mediators of *C. difficile* infection initiation, dissemination, and re-infection, due to their resistance to antimicrobial treatment and disinfection. However, current therapeutics do not directly target sporulation and have no effect on spore formation. Our team has shown that cephamycins, a beta-lactam type antibiotic, can inhibit sporulation by targeting the sporulation specific penicillin binding protein *Cd*SpoVD[1]. SpoVD proteins are highly conserved among spore forming bacteria and play an essential role during sporulation in *C. difficile*[1]. However, our research has identified that sporulation is not inhibited by cephamycins in strains that acquire a sporulation specific protein, *Cd*SpoCR. To understand how *Cd*SpoCR facilitates resistance to cephamycins, recombinant *Cd*SpoCR was produced and assessed for its affinity to a panel of clinical antibiotics. Our research shows that *Cd*SpoCR is unable to bind or has low affinity for many clinically relevant beta-lactams. Here we present the structure of *Cd*SpoCR and discuss the basis of low affinity for beta-lactams.

[1.] Srikhanta, Yogitha N., et al. (2019) “Cephamycins inhibit pathogen sporulation and effectively treat recurrent *Clostridioides difficile* infection.” Nat. Microbiol.4.12:2237-2245

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Mechanisms of immune modulation to support microbiome-based therapeutics

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Michael Abt is an Assistant Professor in the Department of Microbiology at the Perelman School of Medicine, University of Pennsylvania. Michael received his PhD at UPenn and conducted his postdoctoral research at Memorial Sloan Kettering Cancer Center. He received a K99/R00 NIH/NIAID Pathway to Independence Award and joined the UPenn faculty in 2018. His lab's research fuses the disciplines of mucosal immunology, microbial pathogenesis, and microbial ecology to investigate mechanisms of immune homeostasis and protective immunity. Recent work has focused on the contribution of the mucosal immune system to microbiome-based treatment of *C. difficile* infection.

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Clostridioides difficile Toxin B subverts B cell migration, germinal center formation and antibody class switching following vaccination

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Recurrent *Clostridioides difficile* infections (CDI) result in substantial morbidity and mortality. The mechanisms behind these recurrences are unknown, but it is hypothesized that the secreted toxin TcdB, which is the primary driver of *C. difficile* pathogenesis, plays a major role. High affinity TcdB-neutralizing IgG is the best-known correlate of protection against CDI recurrence but is commonly lacking following infection in both mouse models and patients. In murine CDI there is an absence of T follicular helper cell differentiation, reduced IgG class-switching, and B cell memory expansion as well as poor toxin neutralization and a lack of resistance to reinfection. We therefore hypothesized that TcdB suppresses murine IgG recall responses by interfering with germinal center-dependent B cell memory. Treatment of mice with TcdB inhibited IgG recall responses following administration of a booster vaccine leading to reduced toxin neutralization. Histological analyses of draining lymph nodes demonstrated that TcdB severely limited the size and quantity of germinal centers induced by immunization. Following TcdB treatment, CXCR4 transcription was upregulated in the draining lymph node, with CXCR4 surface expression increasing on B cells. *In vitro* B cell migration towards the CXCR4 ligand CXCL12 was elevated in cells obtained from draining lymph nodes of TcdB treated mice. The changes in CXCR4 expression and related migration observed after TcdB treatment were recapitulated in a *C. difficile* infection model. These data demonstrate that *C. difficile* TcdB hijacks reorganization of the secondary lymphoid architecture needed to orchestrate germinal center formation and IgG recall responses.

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Transcriptional landscape of *Clostridioides difficile* infection-induced neutrophilia

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Neutrophils are key first responders in the host response to *Clostridioides difficile infection* (CDI). Excessive tissue and blood neutrophilia is associated with worse histopathology and adverse outcomes. However, the phenotype of CDI-induced neutrophils and mechanisms by which they exacerbate colonic damage remain unknown. Using single-cell neutrophil transcriptomics, we show that CDI leads to transcriptional reprogramming of bone marrow (BM) and blood neutrophils resulting in altered maturation trajectories and amplification of gene expression associated with bactericidal capacity and neutrophil-mediated tissue injury. Sequencing of colonic neutrophils indicates that the hyperinflammatory gene signature is further augmented upon tissue infiltration. We identified that Olfactomedin-4 (*Olfm4*), a glycoprotein expressed in neutrophil specific granules is a highly variable gene among neutrophil sub-populations after infection. The number of $OLFM4^{+}$ neutrophils was increased in mouse colon after CDI, and its protein concentration was elevated in serum of both mice and patients with CDI. Leveraging our transcriptomics data, we found that $Olfm4^{+}$ neutrophils exhibit high expression of genes associated with neutrophil degranulation and activation. Further, we show that: (i) neutrophil stimulation releases OLFM4; (ii) neutrophils from WT mice cause more epithelial injury compared to those from OLFM4 deficient ($OLFM4^{-/-}$) mice; (iii) $OLFM4^{-/-}$ mice had less epithelial damage and better survival after CDI, without effects on pathogen burden; and (iv) rhOLFM4 protein directly exacerbates *C. difficile* toxin-induced epithelial damage. Our study is the first description of neutrophil transcriptional landscape in CDI, and our novel data reveal that a specific neutrophil subpopulation aggravates colonic tissue injury via OLFM4 release.

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Clostridioides difficile infection disrupts host colonic repair

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The toxin-mediated damage caused during *Clostridioides difficile* infection (CDI) is primarily observed in the colon, with significant injury sustained to the intercellular-junctions, polarity determinants and stem cell niches of the colonic epithelial lining. In addition, many patients suffering with CDI readily experience recurrent infection with the bacteria. The effects elicited by CDI during acute infection have been well-reported. However, its impact on colonic regeneration, and the subsequent effects on CDI relapse and extraintestinal complications is less clear. Here we report, via the use of a novel CDI recovery mouse model, that CDI induces substantial damage to the integrity of the colonic lining that takes up to 30 days post-infection to repair, compared to the normal lining turnover rate of 2-3 days. This repair appears to correlate with CDI-mediated disruption of homeostatic colonic stem cells and their regenerative capacity, alongside the activation of damage-induced backup repair mechanisms. Furthermore, we observed the improperly repaired, prolonged leaky gut induced by CDI can trigger inflammation and systemic complications in extraintestinal organs as far as the thymus. Our findings indicate a novel mechanism wherein enteric pathogens may impede the repair process of the host to increase the severity of illness and propagate disease. Such findings provide insight into the impact of enteric infections at and beyond the site of local damage as well as the effectiveness and appropriateness of current treatments, providing foundations for novel investigations into new therapies to enhance recovery and circumvent the reliance on antibiotics.

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POSTER SESSION I

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2 - Deleted in Malignant Brain Tumors 1 (DMBT1) Regulates Mucosal Barrier Function and Epithelial Restitution in *Clostridioides difficile* Infection

Emily Green[1], Hannah Lunnemann[2], Megan Rutherford[2], John Shupe[2], Robert Coffey[2, 3], D. Borden Lacy[2], Nicholas Markham[2, 3, 4]

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Clostridioides difficile infection (CDI) is the most common nosocomial disease in the United States. Risk factors for CDI have been well described, but knowledge of host immune responses during CDI remains incomplete. Mucus is an essential component of immune and barrier properties of the gastrointestinal epithelium, and breakdown of the barrier warrants a proliferative response for epithelial restitution. The relationship between immune function and the regulation of epithelial proliferation during CDI may be tied to specific mucus proteins. Deleted in malignant brain tumors 1 (DMBT1) is a secreted glycoprotein highly expressed at epithelial barrier sites in the gastrointestinal tract. Published data show DMBT1 functions in mucosal immunity and epithelial differentiation. Our preliminary data demonstrate *Dmbt1* expression is upregulated in colonocytes in response to *C. difficile*. We hypothesize DMBT1 promotes mucosal barrier function and regulates epithelial restitution during *C. difficile* infection. Analysis of published single-cell RNA-sequencing data shows *C. difficile*-exposed Apc^{min} mice had a 44.03 log-fold increase in *Dmbt1* expression in differentiated colonocytes at 2-weeks post gavage (*p*-value = 5.00E-193). Immunofluorescence analysis confirms DMBT1 upregulation in mice exposed to *C. difficile* toxin B. The DMBT1 staining is predominantly in mid-crypt colonocytes with cytoplasmic localization and enhanced staining near the apical border. It is not heavily expressed in $MUC2^{+}$ goblet cells. Additionally, the proximal colon contains more DMBT1 staining than the distal colon, particularly in areas of epithelial injury. Defining the regulation and function of DMBT1 is crucial to understanding host mucosal immune response and repair mechanisms during CDI.

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4 - Towards controlled human *C. difficile* colonization and infection

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Clostridioides difficile infection (CDI) is a toxin-mediated gastro-intestinal disease (1). Consequently, much *C. difficile* research focuses on symptomatic individuals and current animal models have a strong focus on symptomatic disease. To address questions related to early colonization events, toxin-independent host and microbiome modulation and immune responses, we wish to establish controlled human colonization and infection models for *C. difficile*.

As a first step, we are setting up a controlled human colonization model using L-NTCD-03, a non-toxigenic clade 4 isolate of *C. difficile*. For L-NTCD-03, extensive characterization using *in vitro* and *in vivo* assays has been performed. We have established quality-controlled small-scale manufacturing of a spore product based on GMP principles, following EMA guidance and recently published principles for production of challenge material (2). We are designing an adaptive dose design study to establish the safety and colonization endpoints of this NTCD strain.

Through extensive discussions with relevant stakeholders within the EU-funded project Inno4vac (3), and based on our experience with L-NTCD-03, we have also selected a clade 1 toxigenic strain, L-TCD-01, for production of challenge material for the controlled human infection model. Setting up this infection model will guided by the results obtained with the NTCD trial.

Both the NTCD and TCD models aim to offer a unique possibility to perform assessment of efficacy of therapeutic interventions and allow early prioritization of lead-compounds.

1. Smits *et al*. Nat Rev Dis Primers. 2016; 2:16020. doi: 10.1038/nrdp.2016.20.
2. La *et al*. Wellcome Trust. 2022. doi: 10.6084/m9.figshare.19411838.v1
3. https://www.inno4vac.eu

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12 - Butyrate induces *Clostridioides difficile* sporulation in vitro

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Short chain fatty acids (SCFAs) are bacterial fermentation end products that contribute to important gut functions such as maintenance of the intestinal barrier, cell signaling, and immune homeostasis. Several studies suggest that the SCFA, butyrate, may be important in alleviating gut infections, including reducing inflammation caused by the healthcare-associated pathogen, *Clostridioides difficile*. Despite this potential, the direct effect of butyrate on *C. difficile* remains unclear. Using an *in vitro* platform, we investigated how SCFAs influence *C. difficile* growth, sporulation, and toxin production. Similar to previous studies, we observed that butyrate supplementation in rich media decreased the growth of *C. difficile* strain 630 in a dose-dependent manner. Attenuated growth is likely dependent on the metabolic environment, as decreased growth was not uniformly observed in minimal media supplemented with different carbohydrates. The presence of butyrate also increased *C. difficile* sporulation and toxin production. RNA-Seq analysis validated our experimental results, demonstrating increased expression of sporulation-related genes in conjunction with alternative metabolic and related *C. difficile* regulatory pathways, such as the carbon catabolite repressor, CcpA. Collectively, these data suggest that butyrate may signal alternative *C. difficile* metabolic and survival strategies, thus modifying its growth and virulence to persist in a changing gut environment.

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14 - Molecular and structural basis of the role of beta-toxin in *Clostridium perfringens* type C enteritis.

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Clostridium perfringens type C causes fatal necrotic enteritis in newborn animals and sporadically in humans. The disease is characterized by rapidly progressing hemorrhage and necrosis of the small intestine. Beta-toxin (CPB), a hemolysin-like β-pore-forming toxin, is the main virulence factor of pathogenic type C strains. It targets endothelial cells in the small intestinal wall leading to vascular damage early during disease progression. We unravelled the molecular and structural basis of CPB target cell specificity. CPB is highly specific towards endothelial and leukocytic cells. Using genome-wide CRISPR-Cas9 loss-of-function screens, CRISPR-Cas9 single gene knockout and ectopic overexpression studies, we unambiguously identified Platelet Endothelial Cell Adhesion Molecule-1 (PECAM1 or CD31) as the cell surface receptor for CPB in endothelial and monocytic cells. We determined the membrane proximal Ig6 domain of CD31 as the toxin binding domain. No other protein receptor candidates could be identified in endothelial cells. The oligomeric pore structure of CPB was resolved using Cryo-EM as symmetrical eightfold protomers, consisting of an N-terminal β-barrel protrusion, a cap, a rim, and a stem domain. The rim domain contains unique loops with surface-exposed charged and aromatic residues that most likely define the receptor specificity of CPB. Structural predictions using alpha fold show that these loops build an ideal binding pocket for the CD31 Ig6 domain. This explains the remarkable receptor and cell type specificity of CPB. Our results correlate with observations in naturally affected pigs and humans and are important to understand the complex pathogenesis of *C. perfringens* type C induced enteritis.

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17 - *Clostridioides difficile* HtrA expression is regulated by its own proteolytic activity and is increased under acidic conditions

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High-temperature requirement A (HtrA) proteins protease/foldases contain a serine-protease domain and one or more PDZ domains. HtrA proteins are important for stress response and survival of bacteria in the host. Consequently, strains that lack a functional HtrA are generally attenuated.

Most - if not all - *C. difficile* genomes encode a single homolog of the serine protease/foldase HtrA.

In contrast to other bacteria, we have previously shown that a *C. difficile htrA* (*cd3284*) knockout is more virulent than wild type in hamsters, likely because of increased toxin levels. The *htrA* knockout also displayed delayed sporulation and decreased binding to CaCo2 cells.

To dissect the relative importance of HtrA's protease *vs.* putative foldase activity for one or more of these phenotypes, we generated *C. difficile* mutant strains in which the proteolytic activity of HtrA was abolished (S217A), in which the PDZ domain was removed or a combination of both.

Strikingly, we found that mutation of the catalytic serine residue led to overexpression of HtrA, as evidenced by W-blot and a reporter assay. Overexpression was not observed when only the PDZ domain was deleted. *In vitro* data with purified HtrA showed that the PDZ domain was not required for proteolytic activity. Together, this shows that proteolytic activity of HtrA is required for regulation of HtrA levels through a transcriptional feedback mechanism.

We have also observed that P*htrA* activity is increased under acidic conditions, but not after exposure to ethanol, high salt or hydrogen peroxide, suggesting a role for HtrA during acid induced stress.

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20 - Comparative genomic of *Clostridioides difficile* pathogenic ribotypes isolated from pet dogs in Rio de Janeiro State

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Clostridioides difficile infection has become more common and severe globally in the previous decade. Because *C. difficile* has been isolated from various animals, a possible zoonotic potential has been raised. The purpose of this study was to compare the genomes of pathogenic ribotypes of *C. difficile* isolated from pet dogs in Rio de Janeiro State, looking for information that provides better understanding of not just the species' zoonotic potential, but also its pathophysiology in dogs. The WGS from five strains were completely sequenced using Illumina MiqSeq system. The generated reads were assembled and annotated with SPAdes algorithm and PROKKA platform, respectively. Following that, a comparative genome analysis was performed to search for single point mutations (SNPs), insertion elements and pathogenic islands that could provide or enhance virulence and antibiotic resistance. In parallel a multilocus sequence typing (MLST) was performed using the 3730XL DNA Analyzer. Our findings show that, despite the fact that strains shared 3366 SNPs in common, each one has distinct traits that set it apart from the others. In a virulence factor analysis, genes linked with adhesion, motility, sporulation, oxidative stress, and heat shock were found in all strains. Concerning to MLST strains belonged to the ST42 and ST15 groups. Our findings corroborate the species zoonotic potential and reveal that *C. difficile* strains that cause CDI in humans can also be isolated from pet dogs and carry virulence and resistance genes.

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22 - *Clostridioides difficile* in foals with or without diarrhea

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Clostridioides difficile is an important pathogen in foals presenting diarrhea. The aim of this study was to compare molecular detection (qPCR) and isolation of *C. difficile* in foals with and without diarrhea up to one year of life. Feces from foals with diarrhea (n=100) and controls (n=100; matched for age, management and farms) were collected and stored at -80°C. Isolation of *C. difficile* followed by PCR targeting specific genes (*tcdA*, *tcdB* and *cdtB*) and subsequent ribotyping of toxigenic isolates was performed. Feces were also subjected to real-time PCR (qPCR) targeting a constitutive gene (16S) and *tcdB*. A total of 36 isolates were obtained (27 diarrheic and 9 control foals, p=0.0009), with 20 of the isolates being toxigenic (16 diarrheic and 4 control foals, p=0.004); 85% of the toxigenic isolates were detected in foals up to 30 days of age (RT126 and 46 were the most common). Considering the 16S qPCR, 59 samples tested positive (39 diarrheic and 20 control foals, p=0.003), with 28 of these samples also testing positive for the toxin B gene (20 diarrheic and 8 control foals, p=0.014). Among the positive samples, 86% were detected within the first 30 days of life. The 16S qPCR exhibited 97% and 85%, and the toxin B qPCR presented 95% and 81% sensitivity and specificity, respectively, when compared to the toxigenic culture. qPCR for *C. difficile* demonstrated good sensitivity and specificity, and foals with diarrhea showed significantly higher rates of *C. difficile* toxigenic isolation than normal foals.

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26 - CRISPRi Phenotyping of Essential Genes in *Clostridioides difficile*

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Clostridioides difficile is a Gram positive, anaerobic, spore forming pathogen that is the leading cause of hospital acquired diarrhea, resulting in about 12,000 deaths per year in the United States. *C. difficile* infections (CDI) are often triggered by broad-spectrum antibiotics that disrupt the normal gut microbiota, and treating CDI with such antibiotics, while initially effective, often leads to relapse. Antibiotics that inhibit *C. difficile* selectively are likely to improve outcomes. In 2015 a Tn mutagenesis screen (Dembek et al., 2015, mBio) identified 404 essential genes in strain R20291. Most of these genes fall into expected categories such as DNA synthesis, transcription or translation. However, many are either hypotheticals or have relatively broad homologies (e.g., transporter) that do not explain why they would be essential. We therefore set out to characterize 194 of these genes by a CRISPRi-based screen with the goal to evaluate essentiality by an orthogonal method and to characterize the terminal phenotype. We hypothesize that changes in cell morphology may allow us to assign genes to functional pathways. Each gene will be targeted by 2 independent sgRNAs for reproducibility. To date we have tested 181 genes. Of these, 113 were confirmed as essential by CRISPRi, 45 had an intermediate viability phenotype, and 23 were clearly not essential. Interestingly, CRISPRi silencing of DNA replication genes led to some of the most profound filamentation phenotypes, suggesting a potential link between DNA replication and cell division, as has been reported in other organisms.

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29 - Investigating the role of *Clostridioides difficile* small, acid-soluble spore proteins during spore outgrowth

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Clostridioides difficile is an anaerobic gut pathogen which relies on its dormant spore form for transmission between hosts. In a host, the spores germinate in response to certain bile acids and amino acids and outgrow into toxin-producing vegetative cells. Spores are resilient to many insults such as chemical treatment, heat exposure, and UV radiation. This resilience is partly conferred by small, acid-soluble spore proteins (SASPs), which are produced during sporulation and make up as much as 20% of the dry weight of a spore. Upon initiation of germination, SASPs are cleaved and rapidly degraded into amino acids. We hypothesize that these amino acids are important as a substrate for the regeneration of cellular NAD^+ through reductive Stickland metabolism, primarily using glycine and leucine due to their high abundance in SASPs. We test this hypothesis through capturing DIC images of outgrowing mutant spores and tracking the length to width ratio of these spores as they progress through outgrowth and into vegetative growth.

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33 - Comparative genomics of disease associated *Clostridium perfringens* isolates

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Clostridium perfringens is the etiological agent for numerous significant animal diseases, such as porcine necrotic enteritis (NE) and small ruminant enterotoxemia. Key to their pathogenicity is their capability to produce a wide array of virulence factors, including enzymes and pore-forming toxins (PFTs). However, our understanding of the virulence machinery associated with *C. perfringens* in different diseases is still limited. To address this issue, we built up a biobank containing *C. perfringens* isolates derived from diagnostic cases with typical signs for clostridial infections. We performed whole-genome sequencing of 136 isolates derived from 89 animals, including pigs, horses, dogs, sheep, goats, alpacas, calves, and lorikeets. Then, we conducted comparative genomics, with a particular focus on pore-forming toxins. The data obtained from our study may provide valuable insights into the genomics of these important veterinary pathogens and help identify central virulence mechanisms used by different strains. Findings of our study will be presented at the conference.

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35 - A Potent and Narrow-Spectrum Antibacterial Against *Clostridioides difficile* Infection

Biruk Birhanu[1], Yuanyuan Qian[1], Jingdong Yang[1], Derong Ding[1], Jeshina Janardhanan[1], Valerie Schroeder[1], Shahriar Mobashery[1], Mayland Chang[1]

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Clostridioides difficile is an anaerobic Gram-positive bacterium that colonizes the gut of patients treated with broad-spectrum antibiotics. A challenge in treating *C. difficile* infection (CDI) is the production of spores that germinate to active vegetative cells in response to host bile acids. The normal gut microflora prevents colonization by *C. difficile* under normal circumstances. Gut microflora dysbiosis by treatment with broad-spectrum antibiotics causes recurrence of CDI in 25% of patients. There are no antibiotics for the treatment of multiple recurrent CDI, which is a dire condition. We report herein that oxadiazole antibiotics exhibit bactericidal activity against *C. difficile* vegetative cells. We screened our existing library of 75 oxadiazoles against vegetative *C. difficile* ATCC 43255. The findings from this library served as the basis for the syntheses of an additional 59 analogs, which were tested against the same strain. We report a potent (MIC_{50} = 0.5 μg/mL, MIC_{90} = 1 μg/mL for 101 strains) and narrow-spectrum oxadiazole (3-(4-(cyclopentyloxy)phenyl)-5-(4-nitro-1H-imidazol-2-yl)-1,2,4-oxadiazole). This oxadiazole is not active against other Gram-positive or Gram-negative organisms, it is bactericidal, and targets cell-wall synthesis.

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39 - Study of a potential antiphage system of the 'abortive infection' type regulated by a non-coding RNA in *Clostridioides difficile*

Marion Saunier[1, 2], Victor Kreis[1], Louis-Charles Fortier[2], Olga Soutourina[1]

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Clostridioides difficile infections are the main cause of antibiotic-associated diarrhea and have drastically increased in the early 2000's due to the emergence of epidemic and hypervirulent strains. Our group identified >200 potential regulatory RNAs in *C. difficile*, which might play important roles in gene expression during *C. difficile* infection. RNA sequencing and Northern blotting led to the identification of a non-coding RNA (ncRNA), specific to the epidemic strain R20291. This ncRNA is present in the conserved phi027 prophage, in an intergenic region upstream of a gene encoding a putative abortive infection protein (Abi). Abi proteins are involved in abortive infection, a defense mechanism against bacteriophages, during which infected bacteria die before the infecting phage can complete its replication cycle. Our goal is to study the function and regulation of this predicted Abi system. Overexpression of the *abi* gene resulted in a growth arrest in liquid and solid medium. Interestingly, co-expression of the ncRNA in *cis* or *trans* showed a restoration of normal growth. Moreover, RT-qPCR experiments on a deletion mutant lacking the ncRNA showed increased expression of the downstream gene, suggesting a negative regulatory effect of the ncRNA on the *abi* gene. Transcriptional fusions with the alkaline phosphatase gene showed a decrease in reporter gene expression in the presence of the ncRNA. The secondary structure of the ncRNA suggests a riboswitch structure but other hypotheses are conceivable as this ncRNA also acts in *trans*. The Abi-like protein could potentially play a role in defense against phages or in prophage maintenance.

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42 - A Conserved Switch Controls Virulence, Sporulation, and Motility in *C. difficile*

Michael DiCandia[1], Adrianne Edwards[1], Cheyenne Lee[1], Ysabella Alcaraz[1], Marcos Monteiro[1], German Vargas Cuebas[1], Pritha Bagchi[1], Shonna McBride[1]

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Spore formation is required for environmental survival and transmission of the human enteropathogenic *Clostridioides difficile*. In all bacterial spore formers, sporulation is regulated through activation of the master response regulator, Spo0A. However, the factors and mechanisms that directly regulate *C. difficile* Spo0A activity are not defined. In the well-studied *Bacillus* species, Spo0A is directly inactivated by Spo0E, a small phosphatase. To understand Spo0E function in *C. difficile*, we created a null mutation of the *spo0E* ortholog and assessed sporulation and physiology. The *spo0E* mutant produced significantly more spores, demonstrating Spo0E represses *C. difficile* sporulation. Unexpectedly, the *spo0E* mutant also exhibited increased motility and toxin production, and enhanced virulence in animal infections. We uncovered that Spo0E interacts with both Spo0A and the toxin and motility regulator, RstA. Direct interactions between Spo0A, Spo0E, and RstA constitute a previously unknown molecular switch that coordinates the regulation of sporulation with motility and toxin production. Reinvestigation of Spo0E function in *B. subtilis* revealed that Spo0E induced motility, demonstrating Spo0E regulation of motility and sporulation among divergent species. Further, we found that Spo0E orthologs are widespread among prokaryotes, suggesting that Spo0E performs conserved regulatory functions in diverse bacteria.

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44 - The Microbiome and Its Restoration to Manage the Impact of Recurrent *Clostridioides difficile* Infection

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Clostridioides difficile infections (CDIs) afflict an estimated 500,000 people in the United States annually, with 30,000 deaths. CDI imposes an economic burden of $5.2 billion per year, with recurrent CDI (rCDI) accounting for $2.8 billion per year. Among patients with CDI, >60% reported psychological and physical symptoms of diminished quality of life. For patients covered by Medicare with 1 episode of rCDI, 27% experienced sepsis and 7% had a colectomy. These rates increased with each subsequent recurrence; among patients with ≥3 CDI recurrences, there was a 24% mortality rate and 43% experienced sepsis. The mechanisms by which the host microbiome modulates CDI complications is not fully understood. The gut microbiome contains trillions of microorganisms, with Bacteroidetes and Firmicutes as the main bacterial organisms in a healthy gut. Standard-of-care antibiotics for CDI and rCDI (vancomycin and fidaxomicin) can further disrupt the composition and/or diversity of the microbiomes, resulting in CDI-associated dysbiosis. Live biotherapeutic products (LBPs) have shown efficacy in preventing rCDI. Fecal microbiota, live-jslm (REBYOTA™, abbreviated here as RBL, previously known as RBX2660), is the first LBP approved by the US Food and Drug Administration to prevent rCDI in individuals 18 years and older following antibiotic treatment for rCDI. In an exploratory analysis, there were shifts in the microbiome towards a healthier composition and diversity and in the metabolome in participants administered RBL. Correcting dysbiosis by restoring the gut microbiome may help reduce the social, clinical, and economic burden associated with CDI and its recurrence.

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46 - The putative flavodoxin FldX and its role in the oxidative stress response in *Clostridioides difficile*

Robert Knop[1], Daniel Troitzsch[1], Silvia Dittmann[1], Thaddäus Echelmeyer[1], Johanna Pukall[1], Lisa Hill[1], Valeria Kamozina [1], Susanne Sievers[1]

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Clostridioides difficile is a vast problem in health care facilities, as it causes serious and recurrent inflammation of the intestinal epithelium. It is considered to be an anaerobic bacterium, but previous studies have shown a high and strain-dependent oxygen tolerance of the pathogen. To understand this tolerance, we aim for identification and characterization of candidates, which are involved in the oxidative stress response of *C. difficile.*

We focused our investigation on flavodoxins - small electron transfer proteins of specifically low potential, featuring a flavin mononucleotide cofactor. An involvement of flavodoxins in the oxidative stress response and their ability to substitute for ferredoxins under iron limitation has been reported for several bacteria but not yet in *C. difficile*.

We investigated the transcriptional induction of seven putative flavodoxins under different conditions, including iron limitation and oxidative stress induced by H_2O_2 and O_2, using Slot Blot and RT-qPCR analyses. In particular, the putative flavodoxin FldX showed a high induction under several of the conditions tested and is thus possibly involved in detoxification of reactive oxygen species. A *fldX* deletion mutant showed strong deficits in growth when exposed to oxidative stress.

To identify the exact function and significance of FldX, we are looking for potential interaction partners using co-immunoprecipitation and pull-down assays. Furthermore, we revealed a membrane-bound localization of FldX using immunogold labelling coupled to transmission electron microscopy. Our results are first steps to a functional characterization of FldX and to the elucidation of its role in metabolism, stress adaptation and virulence of *C. difficile.*

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48 - Synthesis of Muramyl-δ-Lactam in Spore Peptidoglycan of *Clostridioides difficile*

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Clostridioides difficile is a spore-forming human pathogen responsible for significant morbidity and mortality. Infections by this pathogen ensue dysbiosis of the intestinal tract, which lead to germination of the spores. The process of spore formation requires a transition for the cell-wall peptidoglycan of the vegetative *C. difficile* to that of spores, which entails the formation of muramyl-δ-lactam. We describe a set of reactions for three recombinant *C. difficile* proteins, GerS, CwlD and PdaA1, with the use of four synthetic peptidoglycan analogs. CwlD and PdaA1 excise the peptidoglycan stem peptide and the acetyl moiety of *N*-acetyl muramate, respectively. The reaction of CwlD is accelerated in the presence of GerS. With the use of a suitable substrate, we document that PdaA1 catalyzes a novel zinc-dependent transamidation/transpeptidation reaction, an unusual reaction that requires excision of the stem peptide as a pre-requisite.

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50 - A butyrate-centric view of *Clostridioides difficile* pathogenesis

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Clostridioides difficile is a diarrheal pathogen that causes significant human morbidity and mortality worldwide. The primary risk factor for *C. difficile* infection (CDI) is a disrupted microbiome, which is often engendered by antibiotic use. Therefore, strategies that support a healthy microbiome are likely to be important for mitigating *C. difficile* pathogenesis in at-risk individuals. Given that diet is one of the most powerful and easily manipulatable factors that affects the gut microbiome, we hypothesized that diet plays important roles in determining CDI outcomes. Using a mouse model of CDI, we show that mice fed diets deficient in dietary fiber exhibit persistent infection. Conversely, gastrointestinal burdens of *C. difficile* are suppressed in mice fed fiber-rich diets. These effects are not generalizable to all fiber types and effective/ineffective fiber types are differentiated based on whether they lead to elevated production of the short chain fatty acid (SCFA) butyrate by the gut microbiome. We also show that exogenous butyrate is internalized into *C. difficile* cells, is incorporated into intracellular CoA pools, and coincides with alterations in growth and virulence factor expression, together highlighting butyrate as a signaling molecule relevant for its pathogenesis. Our ongoing efforts, aimed at unraveling specific molecular mechanisms of how butyrate and other host-microbiome co-metabolites affect CDI will lead to a better understanding of *C. difficile*, the gut microbiome, and the host immune system. This understanding will be exploited to develop novel interventions (e.g., dietary change, precision probiotics, or new drugs) against *C. difficile* and perhaps other bacterial pathogens.

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52 - Variations in healthy human gut microbiotas confer distinct phenotypes of protection against *Clostridioides difficile* infection

Daniel Marquina[1], Tara Share[1], Gurjit Sidhu[1], Jennifer Golwitzer[1], James Martin[1], Joan Whitlock[1], Eric Li[1], Ajisha Alwin[1], Shannon Roff[2], Gary Wang[1]

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Background: *Clostridioides difficile* infection (CDI) is a leading cause of healthcare-associated infections. Fecal microbiota transplantation (FMT) is an effective treatment for patients suffering from recurrent CDI, yet the connection between donor microbiome and FMT treatment response remains unclear. This study aims to determine the relationship between transplanted donor microbiota and CDI resistance using a humanized gnotobiotic mouse model.

Methods: Groups of otherwise identical germ-free (GF) C57BL/6 mice were orally gavaged with fecal suspension from a cohort of 30 unrelated healthy human donors (*n* = 5 mice for each donor microbiome). After three weeks of colonization, humanized mice were challenged with *C. difficile* VPI 10463 spores (CD). Disease severity, *C. difficile* growth and toxin production, and histopathology were examined. Human donor and murine fecal microbiome were analyzed by 16S rRNA sequencing.

Results: Humanized mice post CD challenge showed four distinct phenotypes - Resistant (asymptomatic, no CD burden or toxins in cecum), Carrier (asymptomatic but detectable CD burden and toxins in cecum), Symptomatic (moderate disease with up to 12% weight loss but 100% survival) and Susceptible (moderate to severe disease with 60-64% mortality). Microbiome analysis revealed specific microbiome features that correlated with each phenotype.

Conclusion: Human fecal microbiome could be classified into resistant, carrier, symptomatic, and susceptible phenotypes in a humanized gnotobiotic *C difficile* challenge mouse model. The distinct phenotypes and their associated microbiome features may facilitate the development of a predictive model to identify the most effective donor microbiome for the treatment of recurrent CDI.

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54 - Identifying a *Clostridioides difficile* bile salt hydrolase

Adegoke Adegbite[1], Joe Sorg[1]

[1]Texas A & M University

Clostridioides difficile spore germination occurs after ingestion in response to certain bile acids (predominantly cholate derivatives). Bile acids are conjugated to taurine or glycine before being secreted into the gastrointestinal tract. Taurocholate has been established has the most potent *C. diff*icile spore germinant. We surprisingly found that *C. difficile* encodes a bile salt hydrolase that de-conjugates taurine-conjugated bile acids and promotes its biofilm formation in vitro. However, *C. difficile* does not encode orthologs of known bile salt hydrolases. To identify this protein, we fractionated lysed *C. difficile* cells using ion exchange and size exclusion chromatography. Fractions with bile salt hydrolase activity were sent for mass spectrometry analysis. Candidates will be confirmed and genes inactivated. The identification of this protein would lead to understanding important mechanisms of *C. difficile* gut colonization and persistence.

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56 - Investigating Glycolipid Synthesis in *Clostridioides difficile*

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The cell envelope of Gram-positive bacteria is often a target for antibiotics, so understanding the biogenesis of the atypical cell envelope of *Clostridioides difficile* provides potential for the discovery of novel drug targets. The membrane of *C. difficile* is composed of 50% glycolipids, which include monohexose-diradylglycerol (MHDRG), dihexose-diradylglycerol (DHDRG), trihexose-diradylglycerol (THDRG), and the unique glycolipid aminohexosyl-hexosyldiradylglycerol (HNHDRG). Previously, we identified an operon, *hexSDF,* that is required for the synthesis of HNHDRG. We hypothesize that HexS adds *N*-acetyl-hexosyl to MHDRG to generate HNHDRG. The enzymes required for synthesis of MHDRG, DHDRG and THDRG are not known. Glycolipid synthesis varies between organisms, some utilize a single glycosyltransferase to processively synthesize mono-, di- and tri-glycolipids while others employ multiple enzymes. Using bioinformatics, we identified putative glycosyltransferases and then constructed mutants of these genes and tested for defects in glycolipid synthesis using thin layer chromatography. We identified two genes encoding putative glycosyltransferases that are required for glycolipid synthesis, *ugtA* and *ugtB*. Our data show that a *ΔugtA* mutant fails to produce any glycolipids, and the *ΔugtB* mutant does not produce DHDRG or THDRG. We propose a model where UgtA synthesizes MHDRG, HexSDF synthesizes HNHDRG from MHDRG, and UgtB synthesizes DHDRG from MHDRG. It is currently unknown if UgtB processively synthesizes THDRG from DHDRG or if another enzyme is responsible. Examining the synthesis pathways of glycolipids in *C. difficile* is important to understanding the biogenesis of the cell envelope and its potential for discovery of novel drug targets to selectively target *C. difficile*.

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60 - Siderophore Utilization in *Clostridioides difficile*

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Clostridioides difficile (*Cd*) is the leading cause of antibiotic associated diarrhea. During colonization, *Cd* must obtain essential nutrients for growth, including iron, which is used both by host cells and bacteria for many cell processes. Very little free iron is available in a mammalian host due to many iron storage mechanisms. Bacterial pathogens have evolved numerous mechanisms for acquiring iron, including small, high-affinity molecules called siderophores. Some bacteria gain a competitive advantage by producing a local pool of siderophore, while others utilize siderophores produced by neighboring bacteria (xenosiderophores). A small subset of *Cd* isolates (74/1894 or 3.9%) encode genes for siderophore biosynthesis. However, those strains without these genes also utilize a variety of siderophores as a sole iron source. *Cd* has three ABC transporters upregulated in iron depleted conditions that are predicted to be involved in siderophore uptake. We purified the siderophore binding proteins from these transporters and tested 11 siderophores for binding using thermal shift. Only ferrichrome caused one siderophore binding protein, FhuD, to unfold at a higher temperature. We made the *fhuDBGC* gene deletion in strain *Cd*630. This strain was unable to utilize ferrichome efficiently in iron limited media (IDM), while complemented *fhuDBGC* restores ferrichrome utilization. As an alternative approach to determine transporter-siderophore specificity beyond FhuDBGC and ferrichrome, we are performing RNAseq on *Cd* grown in IDM plus individual siderophores. This work provides insight into how different Cd isolates compete for nutrients and the role of siderophores in iron acquisition during *Cd* infection.

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64 - Autolysin as a fibronectin receptor on the cell surface of *Clostridium perfringens*

Seiichi Katayama[1], Riyo Aono[2], Shogo Emi[1], Kanako Okabe-Watanabe[3], Hirofumi Nariya[4], Nozomu Matsunaga[1], Yasuo Hitsumoto[1]

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Clostridium perfringens is an obligate anaerobe that causes food poisoning and gas gangrene. *C. perfringens*cells adhere to collagen via fibronectin (Fn) [1]. In this study, we found that a peptidoglycan hydrolase of *C. perfringens*, *i.e.,* autolysin (Acp), has Fn-binding activity. Acp is a 120-kD polypeptide containing 10 cell wall-binding repeats at the N terminus and one catalytic domain (AcpCD) at the C terminus [2]. By a binding assay using recombinant Acp fragments, Fn was found to bind to the AcpCD. Fn binding was significantly decreased in mutant cells lacking Acp (strain 13 *acp::erm*), but was restored by the complementation of the *acp* gene.Three kinds of Fn-binding proteins (FbpC, FbpD, and glyceraldehyde-3-phosphate dehydrogenase) are known in *C. perfringens* [3]. There was no difference in Fn-binding activity between the mutant cells (SAK3) lacking both FbpC and FbpD and the wild-type cells, indicating that these Fn-binding proteins are not involved in Fn binding to *C. perfringens* cells. These results suggest that Acp is an Fn receptor on the surface of *C. perfringens* cells.

[1] Hitsumoto Y, *et al.* Adhesive properties of *Clostridium perfringens* to extracellular matrix proteins collagens and fibronectin. *Anaerobe* 2014. 25:67-71.

[2] Camiade E, *et al.* Characterization of Acp, a peptidoglycan hydrolase of *Clostridium perfringens* with *N*-acetylglucosaminidase activity that is implicated in cell separation and stress-induced autolysis. *J Bacteriol.* 2010. 192:2373-2384.

[3] Katayama S, *et al.* Novel cell wall−associated fibronectin-binding proteins of *Clostridium perfringens*. *Int J Anal Bio-Sci.* 2015. 3: 1-9.

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66 - Surface D-alanylation of *Clostridioides difficile* leads to increased antibiotic resistance

Pierre-Alexandre Lacotte[1], Sandrine Denis-Quanquin[2], Isabelle Martin-Verstraete[3], Thoma Candela[1]

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D-alanylation of surface polysaccharides reduces the affinity and efficacy of cationic antimicrobial compounds (CAMPs) on the bacterial surface. In *C. difficile* (CD), the localization of D-alanylation is unknown and its implication in antibiotic resistance is not elucidated. The aim of our study is to determine the site of D-alanylation in CD and investigate its role in antibiotic susceptibility.

A Δ*dlt* mutant was first constructed. The two major CD polysaccharides, type II polysaccharide (PSII) and lipoteichoic acid (LTA) from the Δ*dlt* mutant and its parental strain were purified. NMR analysis of the polysaccharides revealed the presence of D-alanine on the LTA of the parental strain, but not on the LTA of the Δ*dlt* mutant. In addition, no D-alanine function was identified on purified PSII. Our results therefore highlight the exclusive D-alanylation of LTA in CD. The Δ*dlt* mutant has multiple phenotypes, including greater surface hydrophobicity, increased motility, decreased adherence and increased biofilm formation than the parental strain. In addition, antibiotic susceptibility tests have noteworthy shown increased sensitivity of the Δ*dlt* mutant to bacitracin, teicoplanin and daptomycin. The Dlt1 inhibitor, targeting DltA and already described in *Staphylococcus aureus* and *Enterrococci*, was also tested in CD. Our results support the interest in D-alanylation as a potential therapeutic target in the treatment of CD infections.

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69 - *C. difficile* toxin B induces neurogenic inflammation

John Manion[1, 2, 3], Melissa Musser[4], Gavin Kuziel[3, 5], Min Liu[1, 2, 3], Amy Shepherd[4], Siyu Wang[1, 2, 3, 6], Pyung-Gang Lee[1, 2, 3], Leo Zhao[1, 2, 3], Jie Zhang[1, 2, 3], Ravi K. R. Marreddy[7], Jeffrey Goldsmith[8], Ke Yuan[9], Ralf Gerhard[10], Rongsheng Jin[11], Seth Rakoff-Nahoum[3, 4, 5], Meenakshi Rao[4], Min Dong[1, 2, 3]

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Clostridioides difficile infection (CDI) is a major cause of healthcare-associated gastrointestinal infections. The exaggerated colonic inflammation caused by *C. difficile* toxins such as toxin B (TcdB) damages tissues and promotes *C. difficile* colonization, but how TcdB causes such inflammation is unclear. Here we report that TcdB induces neurogenic inflammation and triggers direct neuropeptide and cytokine secretion. Targeted intoxication of neurons (using toxogenetics) in mice induces neurogenic inflammation in mice subcutaneously and recapitulates colonic histopathology associated with CDI in mice when administered systemically. Conversely, knockout mice lacking the neuropeptides or neuropeptide receptors show reduced pathology in models of both cecal TcdB injection and CDI. Targeting the peripheral nervous system and suppressing neurogenic inflammation thus may provide a broad effective host-oriented approach for treating CDI

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71 - Botulism in waterfowl: retrospective study of autopsy cases submitted to the California animal health and food safety laboratory system (CAHFS) from 1990 to 2022.

Francisco Uzal[1], Sofia Rosales-Martinez[2], Beate Crossley[1], Robert Poppenga[1]

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We reviewed all waterfowl autopsy cases submitted to UCDavis from January, 1990 to August 2022, with a presumptive diagnosis of botulism and tested by mouse bioassay (MBA) for botulinum toxins (BoNTs). 238 carcasses with a presumptive diagnosis of botulism established based on clinical history and signs, coupled with the lack of gross and microscopic changes were received. A diagnosis of botulism was confirmed in 55 (23.11%) cases, while 166 (69.75%) cases were negative and 17 (7.14%) were inconclusive. The most common signs in the MBA positive cases were population die off (85%), weakness (16.36%) and progressive paralysis (12.72%). Samples in which BoNTs were most frequently identified were liver (65.5%) and serum (18.2%). In 53 (96.4%) of the MBA positive cases, type C toxin was found, while the type in the 2 remaining cases was undetermined. Fifteen (80%) of the inconclusive cases and 75 (45.18%) of the negative cases were considered highly suspicious of botulism, because at least one positive bird in the same die off submission, the clinical signs were highly suggestive of botulism, and/or other causes of disease had been ruled out. There are several tests for botulism diagnosis, but the MBA is still the gold standard and remains the most widely used test to detect BoNTs in animals despite its low sensitivity. We are currently working on the development of in-vitro tests with higher sensitivity, and low cost for the detection of BoNT in animals.

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75 - Comparison of microscopic perivascular edema scores between brain regions of sheep with experimental enterotoxemia caused by *Clostridium perfringens* type D wild-type strain CN1020

Francisco Uzal[1], Jorge Garcia[2], Vicki Adams[3], Joaquin Armendano[2], Juliann Beingesser[1], Julian Rood[3]

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C. perfringens type D enterotoxemia is mediated by epsilon toxin (ETX), which causes edema in several organs, including the brain. The aim of this study was to assess whether the severity of brain perivascular edema (BPE) differed in various neuroanatomical locations of sheep with acute enterotoxemia caused by *C. perfringens* type D. We used hematoxylin and eosin-stained sections of brain from 6 sheep that had been intraduodenally inoculated with this pathogen. All sheep developed neurologic signs. Sections of the brain including cerebrum, corpus striatum, thalamus, hippocampus, anterior colliculus, cerebellum, and medulla oblongata were examined histologically. The BPE score of in the examined sections was recorded on a scale of 0 to 3. The proportion of sheep with BPE was 100%. The median BPE scores were compared between brain locations using an aligned rank transformation procedure for nonparametric ANOVAs; multiple comparisons were adjusted using the false discovery rate correction. The median BPE score differed between locations ($p=0.005$). The locations with highest scores were the cerebrum, the anterior colliculus, and the cerebellum. The score in the cerebrum was significantly higher ($p<0.05$) than in the cerebellum, thalamus, anterior colliculus, and medulla oblongata. Our results indicate that even though BPE can be widespread in the brain of sheep with experimental acute type D enterotoxemia, from a diagnostic standpoint, sampling and examining the white matter of the cerebrum, cerebellum and anterior colliculus provides better chances of finding higher scores of BPE, which is a pathologic hallmark of type D enterotoxemia in sheep.

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81 - A VirS receptor decoy peptide that binds the Agr signal peptide can attenuate gas gangrene lesions caused by *Clostridium perfringens* type A strain ATCC3624

Francisco Uzal[1], Fabio Mendonca[2], Bruce McClane[3], Jihong Li[3]

[1]UCDavis, [2]Federal Rural University of Pernambuco, Brazil, [3]University of Pittsburgh

Clostridium perfringens type A causes gas gangrene in humans and animals due to the action of alpha toxin (CPA) and perfringolysin O (PFO). Binding of the Agr signal peptide (SP) to the VirS membrane sensor regulates production of both toxins. We previously showed that a synthetic peptide named KIGK, which corresponds to the SP-binding region of VirS, interferes with in vitro production of beta toxin by type B and C strains while KIGKD, which is KIGK with a N to D substitution, did not exert this effect. The current study evaluated whether these two synthetic peptides might similarly affect PFO or CPA production in vitro or the development of gas gangrene in a mouse model. KIGK, but not KIGKD, reduced CPA and PFO production by type A strain ATCC3624 in vitro. When ATCC3624 with or without KIGK or KIGKD were injected intramuscularly in the left thigh of Balb/c mice, similar numbers of viable *C. perfringens* were recovered from all mice. Gross changes in mice receiving ATCC3624 with or without KIGKD consisted of swelling, edema, and hemorrhage. No gross lesions were observed in mice receiving *C. perfringens* plus the KIGK peptide. Microscopically, mice receiving *C. perfringens* had severe muscle degeneration/necrosis and inflammation. Mice receiving *C. perfringens* KIGKD presented moderate to severe histological lesions, while mice receiving *C. perfringens* + KIGK presented only mild lesions. These results provide *in vivo* evidence that VirS-based receptor decoy inhibitory peptides can attenuate *C. perfringens* type A gas gangrene.

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83 - DNA methylation promotes *Clostridioides difficile* sporulation by enhancing transcription of a gene encoding the cell fate determinant SpoIIE

Pola Kuhn[1], John W. Ribis[1], Shailab Shrestha[1], Aimee Shen[1]

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Many bacteria use DNA methylation as a mechanism to epigenetically regulate diverse cellular processes such as chromosome replication, DNA repair, and transcriptional activation. This post-replicative modification can result in phase-variable gene expression, leading to phenotypic cell variants with distinct functions or cellular fates. While recent advances in methylome sequencing have revealed that DNA methylation is ubiquitous in bacteria, the exact mechanism by which it epigenetically regulates phenotypic heterogeneity remains unclear in most cases. We recently determined that methylation by the *Clostridioides difficile*-specific DNA methyltransferase CamA promotes spore formation. Since the formation of aerotolerant spores is critical for this enteric pathogen and obligate anaerobe to transmit disease, we sought to elucidate the mechanism by which DNA methylation enhances sporulation. Using transcriptional reporters, RNA-Seq, and qRT-PCR, we show that CamA promotes the transcription of *spoIIE*, which encodes a regulator of asymmetric division that also functions to activate the earliest acting sporulation-specific sigma factor σ^F. Notably, the inactivation of a single CamA methylation site in *spoIIE*'s promoter region reduces both *spoIIE* expression and the frequency of sporulation. Since the methylation site overlaps with the binding site of the master transcriptional regulator Spo0A, which directly activates *spoIIE* transcription, we propose a model in which DNA methylation increases the binding affinity of Spo0A for the *spoIIE* promoter. Given that SpoIIE activation commits *B. subtilis* cells to completing sporulation, our data suggest a mechanism by which CamA-mediated methylation regulates cell fate and enhances disease transmission.

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86 - CspA:CspC heterodimer formation is conserved across clostridial family members and could play an essential role in germinant and co-germinant signaling

Juan Antonio Serrano Jimenez[1], A. Ioana Stoica[1], Emily R. Forster[1], Aimee Shen[1]

[1]Tufts University School of Medicine

Clostridial pathogens, as obligate anaerobes, rely on spores as their primary transmissive form, which exhibit remarkable resistance to extreme environmental conditions. Accordingly, germination is critical for these pathogens to initiate disease. Germination in spore-forming bacteria is induced by germinants, and its sensing by germinant receptors initiates a signaling pathway that leads to the degradation of the protective cortex layer, which is essential for spore germination. In many clostridial organisms, the cortex-degrading enzyme, SleC, must be proteolytically activated through the action of three subtilisin-like serine protease family members: CspA, CspB, and CspC. Originally identified in *Clostridium perfringens*, each of these proteases is essential for the activation of SleC. In contrast, in *Clostridioides difficile*, CspA and CspC are pseudoproteases that play key roles in sensing germinant (bile acid) and co-germinant signals (Ca^{2+}/amino acids). *C. difficile* CspA and CspC are needed to activate CspB, the active protease responsible for SleC cleavage. We recently discovered that CspA and CspC form a stable heterodimer that is likely essential for germination signaling. Intriguingly, we determined that CspA:CspC complex formation is conserved among other members of the Peptostreptococcaceae family, as well as the Clostridiaceae family. While the functional significance of the CspA:CspC complex in *C. perfringens* is unclear, it may explain why the three proteases are not functionally redundant. Since recent studies show that *C. perfringens* spores germinate in the presence of bile acids and amino acids, the CspA:CspC heterodimer may control *C. perfringens*'s response to these small molecule signals analogous to *C. difficile*.

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88 - Heterogeneity of neutrophils in patients with *Clostridioides difficile* infection (CDI)

Girija Ramakrishnan[1], Mary Young[1], William Petri[1]

[1]University of Virginia

Neutrophil homeostasis is influenced by multiple factors including signals deriving from infection. We hypothesize that disease severity in CDI is reflected in the developmental features of peripheral blood neutrophils. As part of an ongoing study, we have stored fixed leukocytes from blood collected within 24 hours of diagnosis from a cohort of hospitalized patients with acute CDI (N=66, 51% female, recurrent CDI=26%). We also have stored blood cells from recurrent CDI patients at time of and two months after Fecal Microbial Transplant (FMT) therapy (N=18). Using healthy control samples, we first established that neutrophils in stored blood are sufficiently well preserved for phenotyping by flow cytometry using surface markers CD66b, CD11b, CD16 and CD10. Neutrophil counts by flow cytometry correlated well with those from Complete Blood Counts (CBC) of fresh blood ($R^2 = 0.986$). $CD66b^+$ neutrophil representation in blood cells was significantly higher ($P<0.0001$) in 21 stored CDI samples (median=81.55%) compared to 15 healthy controls (median=52.3 %). CD10 surface staining of the CDI samples was heterogeneous with overall lower representation of mature $CD10^+$ neutrophils (mean=64.39 ± 30.07%) compared to healthy controls (mean=95.09 ± 2.07%; $P<0.0001$). We were able to recover RNA from fixed blood cells for characterization of gene expression. As a first test, we successfully used RT-qPCR to detect neutrophil-specific transcripts: *camp*, *slpi* and *S100A12* in blood cells. Our ongoing analysis of gene expression in neutrophils following selective immunoaffinity purification from fixed blood cells may shed light on neutrophil developmental pathways that impact disease severity, relapse and recovery from CDI.

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90 - Acidification-dependent suppression of *C. difficile* by pathogenic and commensal enterococci in vitro

Holly Smith[1], Alicia Wood[1], Angus Johnson[1], Avi Stern[1], Lesly-Hannah Gutierrez[1], Zainab Sikander[1], Addelis Agosto[1], Peter McKenney[1]

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Clostridioides difficile and Vancomycin-resistant *Enterococcus faecium* (VRE) are commonly co-isolated from hospitalized patients. We sought to develop in vitro co-culture systems to identify interactions and study biofilm formation by these two opportunistic pathogens. We found that VRE quickly acidifies media containing glucose, fructose and trehalose to a pH that is not compatible with growth of *C. difficile*. Acidification was necessary, sufficient and the dominant method of VRE-mediated suppression of *C. difficile*. This mechanism is conserved among a panel of pathogenic and commensal enterococci and Clostridia and was maintained when strains were co-cultured in ex vivo in cecal contents from germ free mice. Robust co-culture conditions and biofilm formation were achieved by utilizing a carbon source that is not acidified by VRE. Co-culture VRE-*C. difficile* biofilms using fucose as a carbon source were increased in thickness, suggesting tolerance between the two species under these conditions.

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92 - Control of a sporulation checkpoint by a two-component signaling system in *Clostridioides difficile*

Megan Kempher[1, 2], Tyler Shadid[2], Savannah Morris[1], Jimmy Ballard[2], Ann West[1]

[1]University of Oklahoma , [2]University of Oklahoma Health Sciences Center

Bacteria use two-component signal transduction systems (TCSs) to monitor fluctuations in their environment and control cellular responses to changing conditions. TCSs consist of a sensor histidine kinase (HK) and a cognate response regulator (RR) protein. Upon sensing certain environmental stimuli the HK autophosphorylates at a histidine residue. The phosphoryl group is subsequently transferred to an aspartate residue in the RR inducing a conformational change that alters the activity of the output response, which is often transcriptional regulation. *Clostridioides difficile* is a Gram positive, anaerobic pathogen that is a leading cause of nosocomial infections in the US. *C. difficile* produces metabolically dormant spores that are the primary mediator of transmission between hosts. We recently identified a RR (CD1688) in *C. difficile* that negatively regulates spore production. A Δ*cd1688* strain produced ~3.5 fold more spores than the wildtype (WT) 630 strain and was accompanied by increased expression of sporulation specific sigma factors. Fluorescent microscopy determined asymmetric division, the first developmental step in sporulation, occurred earlier and in a higher percentage of cells in the *C. difficile* Δ*cd1688* strain compared to WT. Further work determined CD1688 directly regulates the expression of *spoIIR*, which encodes a signaling protein that temporally and spatially coordinates the distinct transcriptional programs that occur in the mother cell and forespore during sporogenesis. Overexpression of *spoIIR* mimicked the hypersporulation phenotype observed in the *C. difficile* Δ*cd1688* strain. Taken together, our data suggests regulation of *spoIIR* is a crucial checkpoint controlling progression through the sporulation pathway.

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95 - Within-person evolution following engraftment of a defined probiotic in the human gut

Tami Lieberman[1], Alyssa Haynes[1]

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Understanding the selective forces and extent of within-person evolution that commensal gut microbes experience within individual hosts is important to the development of engraftable microbiome therapeutics. Species of the genus *Bacteroides* have been shown to evolve via *de novo* mutation within individual gut microbiomes, but the role of within-person evolution in other species exists and the extent to which it is person-specific remains unknown. Vedanta Bioscience's VE303 Phase II clinical trial data offer a unique opportunity to study the evolution of the *Clostridia* in human gut microbiomes, and enables the study of evolution of the same strain across different people. VE303 is a defined probiotic consortium of eight commensal *Clostridia* strains from clusters IV, XIVa, and XVII, demonstrated to engraft effectively in recipients. Given the defined background genotype of each strain in the product, we are able to alleviate the challenge of linking a *de novo*mutation to its ancestral genotype. Here, I will present preliminary results identifying *de novo* mutations that emerged within VE303 strains across 30 subjects with sustained engraftment 168 days post-treatment. Through characterizing markers of person-specific adaptation and within-host parallel evolution, we aim to gain a fuller understanding of the diversity and universality of selective pressures on commensal *Clostridia* in human gut microbiomes.

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97 - Nonsteroidal anti-inflammatory drugs exacerbate *Clostridioides difficile* infection

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Clostridioides difficile is the most reported nosocomial pathogen and is an urgent public health threat. This bacterium infects the colon, causing damage to the colonic mucosa through the action of two potent exotoxins. Factors shaping *C. difficile* pathogenesis are incompletely understood but are likely due to the ecological factors in the gastrointestinal ecosystem, mucosal immune responses, and environmental factors. Emerging evidence suggests that previously unappreciated environmental factors, such as diet and pharmaceutical drugs, influence disease manifestation. Nonsteroidal anti-inflammatory drugs (NSAIDs) are one of the most used pharmaceutical drugs in the world and function by targeting cyclooxygenases (COX) enzymes. We recently demonstrated that NSAIDs worsen the course of *C. difficile* infection (CDI). Mice infected with *C. difficile* and pretreated with NSAIDs showed dramatic exacerbation of disease. To dissect the mechanism of NSAIDs effects during CDI, I have developed an in vivo and in vitro system using mice and colonic epithelial cells (CECs). First, I have shown that NSAID treatment followed by *C. difficile* intoxication in CECs increases permeability and inflammatory cell death. NSAIDs are drugs that inhibit COX enzyme; however, data suggest they can have off-target effects on the mitochondria. My data demonstrate that NSAIDs uncouple mitochondrial functions during CDI, a phenomenon independent of COX enzyme inhibition and the downstream-produced molecules prostaglandins. In vivo mitochondrial uncouplers phenocopy NSAIDs and lead to an increase in mortality, disease severity, and CEC damage. Moreover, I have shown that NSAIDs disrupt colonic epithelial mitochondrial functions by increasing mitochondrial damage, superoxide production, and decreasing membrane potential.

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99 - The Sympathetic Nervous System is Necessary for Disease Pathology in *C. difficile* Infection

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Clostridioides difficile infection (CDI) is a prevalent hospital-acquired infection with a broad spectrum of symptoms, ranging from diarrhea to fatality. The biological determinants contributing to disease severity remain unclear and the conventional approach to CDI treatment, antibiotics, significantly increases the risk of recurrent infection. Thus, our lab examines alternative therapeutic approaches that spare protective gut microbial communities. Extensive literature suggests that the sympathetic nervous system (SNS) plays a significant role as an immunomodulator. We found through both pharmacological ablation of peripheral SNS neurons (with 6-OHDA) and inhibition of norepinephrine synthesis (with nepicastat) that noradrenergic neurons are necessary for disease phenotypes in both R20291 (CDT-expressing) and VPI 10463 (non CDT-expressing) strains. Additionally, neutrophil recruitment (36.76 +/- 5.23% vs 14.06% +/-5.9% of CD45+ cells; $p < 0.001$) and IL-1β (6688 pg/mL vs 325.9 pg/mL +/- SE of difference 1019; $p < 0.001$) is diminished in mice treated with 6-OHDA. Following, pharmacological inhibition of alpha 2 adrenergic receptors (a2ar; with inhibitor RX 821002), reduces the mortality rate in CDI mice (20% mortality vs 100% in control; $p < 0.0001$). We did not find improved outcomes in CDI disease with beta adrenergic receptor ($p<0.05$ but increases mortality rate) or alpha 1 adrenergic receptor blockers($p=0.10$). Together these data demonstrate that adrenergic signaling through the a2ar is necessary for CDI disease pathology in mice and suggest that SNS neurons activate the inflammasome during infection.

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101 - Transcriptional landscape of *Clostridioides difficile* infection-induced neutrophilia

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Neutrophils are key first responders in the host response to *Clostridioides difficile infection* (CDI). Excessive tissue and blood neutrophilia is associated with worse histopathology and adverse outcomes. However, the phenotype of CDI-induced neutrophils and mechanisms by which they exacerbate colonic damage remain unknown. Using single-cell neutrophil transcriptomics, we show that CDI leads to transcriptional reprogramming of bone marrow (BM) and blood neutrophils resulting in altered maturation trajectories and amplification of gene expression associated with bactericidal capacity and neutrophil-mediated tissue injury. Sequencing of colonic neutrophils indicates that the hyperinflammatory gene signature is further augmented upon tissue infiltration. We identified that Olfactomedin-4 (*Olfm4*), a glycoprotein expressed in neutrophil specific granules is a highly variable gene among neutrophil sub-populations after infection. The number of OLFM4^{+} neutrophils was increased in mouse colon after CDI, and its protein concentration was elevated in serum of both mice and patients with CDI. Leveraging our transcriptomics data, we found that *Olfm4*$^{+}$ neutrophils exhibit high expression of genes associated with neutrophil degranulation and activation. Further, we show that: (i) neutrophil stimulation releases OLFM4; (ii) neutrophils from WT mice cause more epithelial injury compared to those from OLFM4 deficient (OLFM4$^{-/-}$) mice; (iii) OLFM4$^{-/-}$ mice had less epithelial damage and better survival after CDI, without effects on pathogen burden; and (iv) rhOLFM4 protein directly exacerbates *C. difficile* toxin-induced epithelial damage. Our study is the first description of neutrophil transcriptional landscape in CDI, and our novel data reveal that a specific neutrophil subpopulation aggravates colonic tissue injury via OLFM4 release.

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105 - Microbial and Host Factors that Modulate Differences in Host's Clinical Outcome to *Clostridioides difficile* Infection

Armando Lerma[1], Thomas Auchtung[1], Jennifer Auchtung[1]

[1]University of Nebraska-Lincoln

Clostridioides difficile is one of the most important pathogens in hospital and community healthcare settings. The clinical outcome of infection of toxigenic *C. difficile* infection (CDI) can fall within a wide range of disease severity from asymptomatic colonization to fulminant pseudomembranous colitis and death. In recent studies, it has been suggested that a high proportion of nosocomial CDI cases are transmitted from asymptomatic carriers which might be acting as infection reservoirs. Investigating what causes the different responses to infection could lead to the development of novel prevention and treatment strategies. Although several explanations have been proposed to explain variations in susceptibility, understanding of the exact mechanisms that underlie the spectrum of variation in CDI disease severity remains limited and further research is needed to determine what factors are responsible for these variations. In this work, we establish different human microbiota-associated (HMA) mouse models. By analyzing innate immune responses to CDI, we demonstrate that these models reproduce differences in disease severity during infection that were largely based on mouse strain and independent from *C. difficile* burden or toxin activity. Altogether, our ^{HMA}mouse models demonstrated the potential to study interactions between microbiome, pathogen and host inflammatory responses in the context of CDI.

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107 - *C. difficile*: Spore no more!

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The opportunistic anaerobe, *C. difficile,* is responsible for most cases of hospital-acquired, antibiotic-associated diarrhoea. *C. difficile* infections (CDI) occur after disruption of gut microbiota by broad-spectrum antibiotics. Treatment of CDIs is currently a further course of antibiotics, which enhances gut dysbiosis.

C. difficile can form highly resistant spores which allow transmission via the faecal-oral route, and lead to recurrent infections. Sporulation initiates with asymmetric cell division, followed by engulfment of the forespore by the mother cell, spore maturation and mother cell lysis for release of the mature spore. Here we report the recent developments in our study of peptidoglycan hydrolases SpoIID and SpoIIP, required for remodelling the peptidoglycan during engulfment and part of the engulfasome machinery. Previously determined SpoIID structure and activity showed requirement for zinc, but the role of the metal has not been determined. Current work involves purification and crystallisation of SpoIID as well as catalytic and zinc-binding mutants to further investigate protein activity and specificity. We also aim to identify novel binding sites using fragment screening, to further develop novel species-specific CDI therapeutics. A similar approach has been extended to SpoIIP to further elucidate its function and determine its structure.

Understanding the proteins involved in engulfment is pivotal to prevent sporulation and thus reduce transmission and recurrent CDIs.

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111 - High-throughput methodology for reproducible growth curves in *Clostridioides difficile*

ThanhPhuong Le[1], Eugénie Bassères[1], Taryn Eubank[1], Jahangir Alam[1], Khurshida Begum[1], Anne Gonzales-Luna[1], Chris Lancaster[1], Kevin Garey[1]

[1]University of Houston

Background: *Clostridioides difficile* infection causes a wide spectrum of symptoms partly attributed to the fitness of the infecting strain. However, there is not a well-established high-throughput assay that assesses *C. difficile* fitness using growth curves. The purpose of this study was to develop a high-throughput technique to obtain reproducible *C. difficile* growth curves.

Methods: Fifty-three clinical *C. difficile* isolates (17 ribotypes) were included. Growth curves were performed (in 24-well plate) in duplicate over 24h using Cytation Imaging Reader with 5% oxyrase in BHI media. R package Growthcurver summarized the growth characteristics to compute the empirical area under the curve (AUC_E) and the exponential growth rate (r). The reproducibility of this methodology was assessed using the coefficient of variation (CV%).

Results: *C. difficile* strains exhibited distinct growth patterns over time with wide variability in AUC_E (mean±SD; Range: 6-17). AUC_E for each individual isolate was reproducible with CV% ≤ 30% (52/53). One outlier exhibited a slightly higher CV% of 32%. The AUE_E varied among isolates within the same ribotype and no association was observed between ribotype and AUC_E. The initial population size (n0) did not affect the exponential growth rate (r). Growth rates for each isolate were different by ± 0.2 with the exception of 11 isolates which exhibited a difference of ± 0.3.

Conclusion: The developed assay provided a high-throughput and reproducible growth curves for tracking *C. difficile* growth over 24 hours. This assay provides a reliable methodology for future investigations that aim to assess phenotype-associated fitness costs using growth curves.

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113 - Macrophage Migration Inhibitory Factor (MIF) Kinetics in *Clostridioides difficile* Infection

Ann Mathew[1], Alexander Huber[2], Shinsmon Jose[2], Kristin Weghorn[2], Maggie Powers-Fletcher[2], Rajat Madan[2], Rowis Sous[2]

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Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine produced in response to various infectious insults. We have previously shown a key role for MIF in *Clostridioides difficile* infection (CDI): patients with CDI had significantly higher circulating MIF than controls, and MIF neutralization in a mouse model improved host survival. Although these data suggest that targeting MIF has therapeutic potential, the kinetics of MIF production and the factors that influence its release remain unknown. Using a patient cohort from UC Medical Center, we found that after CDI, the highest plasma MIF concentration was present during the pre-diagnosis phase (day -2 and day -1). Notably, blocking of MIF after *C. difficile* challenge in the preclinical mouse model was less effective compared to the pre-challenge administration of neutralization antibody. In a separate cohort of CDI patients, we show that host genetics has a critical impact on CDI-induced plasma MIF concentration; wherein individuals homozygous for the recessive allele of a common SNP in the leptin receptor had significantly higher plasma MIF compared to individuals homozygous for ancestral allele. Altogether, our data reveal that ***(i)*** MIF is an acute phase cytokine in CDI whose systemic levels are influenced by host genetics; and ***(ii)*** blocking MIF during acute phase of CDI can improve survival in pre-clinical models, suggesting that MIF is a potential target for novel CDI therapeutics. Our studies are now focused on elucidating the mechanisms of MIF production and release in CDI, and understanding how the *Lepr* SNP enhances CDI-induced MIF.

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116 - A novel family of small proteins is regulated by the second messengers c-di-GMP and c-di-AMP in *Clostridioides difficile*

Adriana Badilla Lobo[1], Frédéric Barbut[2], Olga Soutourina[1], Johann Peltier[1]

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Pathophysiology of the human enteropathogen *Clostridioides difficile* is controlled by complex regulatory networks, including RNA-based mechanisms such as riboswitches. Riboswitches are located at the 5' untranslated end of mRNAs and bind ligands triggering a conformational change that positively or negatively affects the expression of the downstream coding sequence. Sixteen riboswitches responding to the second messenger c-di-GMP are present in *C. difficile*. In this study, we identified that five of them are located upstream of putative genes encoding almost identical small proteins (SP). Detection by immunoblotting of a tagged SP derivative provided evidence for its synthesis and its localization in the cytosol. RNA-seq analyses showed a decrease of the five SP transcripts in response to not only c-di-GMP but also to the second messenger c-di-AMP. Furthermore, reporter assays measuring transcriptional regulation of an SP gene in different strain backgrounds revealed that c-di-GMP modulates gene expression through interaction with the riboswitch, whereas c-di-AMP regulates the promoter activity. Overexpression of one of the SP genes resulted in growth defect and hypersporulation. In agreement with the latter phenotype, RNA-seq on the SP overexpressing strain revealed the upregulation of many SigG and SigK-dependent transcripts. Further, we recently generated a strain deleted of all five SP-encoding genes, and the phenotypic characterization of this strain, with an emphasis on sporulation, is underway. Altogether, our data suggest that the genes encoding this new family of small proteins are regulated by both c-di-GMP and c-di-AMP to modulate *C. difficile* spore formation.

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118 - *Clostridioides difficile* proline metabolism contributes to colonization human fecal communities cultured in minibioreactor arrays

Xiaoyun Huang[1], April Johnson[1], Thomas Auchtung[1], Hugh McCullough[1], Armando Lerma[1], Thomas Horvath[2], Anthony Haag[2], Jennifer Auchtung[1]

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The GI microbiota plays an important role in limiting *Clostridioides difficile* colonization. The microbiota can stimulate the immune system, alter bile acid pools, compete for nutrients, and/or produce metabolites that inhibit *C. difficile*. We previously used *in vitro* minibioreactors to investigate how different *C. difficile* strains are able to colonize human fecal microbial communities and to identify simplified communities able to inhibit *C. difficile* colonization. To better understand how microbiota composition and function influence *C. difficile* susceptibility in our model, we tested *C. difficile* susceptibility across microbial communities established from twelve different healthy people; susceptibility was tested in the absence of antibiotic or following treatment with six different antibiotics (Augmentin, azithromycin, cefaclor, ceftriaxone, clindamycin, and fidaxomicin). While all antibiotics disrupted microbiota composition, only clindamycin-treatment led to robust *C. difficile* colonization. Analysis of levels of the bile salts taurocholate, cholate, and deoxycholate in resistant and susceptible communities demonstrated no correlation between bile salt levels and susceptibility to infection. Because preliminary studies demonstrated that amino acids were partially depleted by growth of human fecal microbial communities, we hypothesized that *C. difficile* colonization in our model may be dependent upon its ability to metabolize amino acids through Stickland metabolism. To test this hypothesis, we compared the ability of a *C. difficile* mutant unable to metabolize proline (*prdB*) to a wild-type strain. We observed that proline metabolism was important for persistence in a subset of microbial communities. Future studies will examine how microbiota composition impacts *C. difficile* occupation of different nutritional niches.

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121 - Ibezapolstat modulates *Clostridioides difficile* virulence factors in vitro

Eugénie Bassères[1], Khurshida Begum[1], Chenlin Hu[1], Kevin Garey[1]

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Background

Ibezapolstat is a Gram-positive selective spectrum antibiotic in phase 2 clinical trials for *C. difficile* infection. With a unique mechanism of action that targets the DNA pol IIIC enzyme, IBZ may demonstrate unique pharmacologic properties beyond bacterial killing. The goal of this study is to assess its impact on *C. difficile* virulence properties.

Methods

C. difficile reference strains CD630 and R20291 were treated with ibezapolstat from subinhibitory to minimum inhibitory concentrations of ibezapolstat. Toxin A and B concentrations were measured by ELISA (tgcBiomics). Expression of flagellar genes fliA, flgB and fliC was assessed after 4h treatment of ibezapolstat by RTqPCR (CD630 only). Morphology changes induced by ibezapolstat were evaluated by bright field microscopy at 10-40X magnification.

Results

Toxin A and B concentrations were decreased by ibezapolstat in a dose-dependent manner. Toxin production was normalized to controls with a reduction of toxin levels of 55% (CD630) to 60% (R20291) was shown. A decrease of motility and flagellar genes up to 50% was observed after 4h treatment at 0.25xMIC. Cell phenotype demonstrated elongated cells at sub-inhibitory to MIC for both strains.

Conclusion

Ibezapolstat treatment affects virulence determinants of *C. difficile in vitro*. Further mechanistic research is needed explain this unique selectivity profile.

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123 - Tracking bacterial endospore dispersal in wastewater through dipicolinic acid quantification

Jayne E. Rattray[1], María Bautista[1], Jangwoo Lee[1], Emily Au[2], Chloe Papparis[1], Janine McCalder[1], Thomas Louie[3], Kevin Frankowski[4], Michael D. Parkins[2], Casey R.J. Hubert[1]

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Wastewater-based surveillance offers an objective, comprehensive and inclusive way to understand the prevalence of microorganisms sourced from defined human populations. Bacterial endospores are resilient, dormant structures created by certain members of the phylum *Firmicutes*. Sporobiota (bacteria capable of forming endospores) result in *Firmicutes* being the most abundant phylum in gut microbiomes, consisting of *Clostridium* and ~200 other genera. Endospores aid survival when these populations encounter unfavourable environmental conditions, facilitating large-scale passive dispersal. The human gut contains 10^{13} to 10^{14} microorganisms making it one of the most densely populated environments on Earth such that sporulation by gut bacteria exiting the digestive tract may represent a significant transport vector for horizontally acquired genes. Dipicolinic acid (DPA) lowers the water content of spores and contributes a major portion of spore dry weight, e.g., 5-15% in *Clostridium* species. Molecular quantification of DPA using HPLC-fluorescence and Tb^{3+} chelation revealed abundances on the order of 10^5 to10^6 endospores mL^{-1} in municipal and hospital wastewater. Endospore levels strongly correlated with qPCR measured abundance of *C.difficile* gene markers (i.e., *C. difficile* specific 16S-rRNA genes and *tcdA/B*, using human 18S rRNA genes for normalization). However, an inverse relationship was observed between endospores and general bacterial load (i.e., qPCR of total 16S rRNA and *Bacteroides* HF183 genes). These preliminary results suggests an association between endospores and vegetative *C.difficile* cells and toxin B, but not with the rest of the bacterial population. DPA analysis in wastewater offers a promising method for tracking the dispersal of endospores derived from human gut microbiomes.

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125 - Development of a mucosal vaccine against *Clostridioides difficile* infections

I-Hsiu Huang[1], Yu-Shan Lin[2], Jun-Jia Gong[2], Joseph McCreary[1], Jenn-Wei Chen[2]

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Clostridioides difficile (*C. difficile*) is a gram-positive, obligate anaerobic spore-forming bacteria that is the major cause of antibiotic-associated diarrhea. The primary risk factors for *C. difficile* infection (CDI) are antibiotic therapies that disrupt the hosts' normal microbiota and modify the endogenous gastrointestinal flora. The current treatment of CDI is mostly based on the usage of antibiotics but growing incidences of treatment failure or multiple relapses have raised concerns about the need for alternative therapies. In this study, we evaluated the potential of developing mucosal vaccines via either the oral or the nasal route. For oral delivery, we demonstrated that the encapsulation of protein antigens using biodegradable nanoparticles. Our preliminary results demonstrated that orally delivered protein antigens induced robust antibody responses and protected mice from C. difficile infections. Nasal delivery were proven to generate antigen-specific antibodies albeit the protective effect were less robust. Lastly, we tested the hypothesis that a chimeric protein construct consisted of a fragment of toxin B linked to a cell surface protein would provide protection against CDIs in a mice model of infection.

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127 - Comparative genomic analysis of *C. difficile* strains from Mexico and around the world

Claudia Martinez De La Peña[1], Charlen Ortiz Flores[2], Alba Romero Rodriguez[3], Thomas Louie[4]

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Clostridioides difficile infection (CDI) is a public health associated with severe disease and increased mortality throughout developed countries. This increase is caused by a worldwide spread of epidemic strains such as B1/NAP1/RT027/ST01, which has high virulence and antibiotic resistance. In Mexico, previous studies have shown the presence of this and other hypervirulent strains, emphasizing the importance of studies that investigate differences and similarities among *C. difficile* strains. The objective of this study was to analyze the genomic and pathogenic characteristics of *C. difficile* strains isolated from Mexican patients and perform a comparative genomics analysis with other strains from Canada and other countries. Thirty-seven *C. difficile* strains were isolated from patients from Puebla Mexico during June to December 2022. The *tpi, tcdA, tcdB*, *cdtA* and *cdtB* genes were amplified by PCR. Susceptibility to Vancomycin and Metronidazole were tested, finding resistance to vancomycin in 4 strains and resistance to metronidazole in 9 strains. WGS of 10 Mexican strains was carried out and compared with sequences from Canada and other countries. A high prevalence of the TcdB toxin was found in the Mexican strains. Differences in antibiotic resistance genes were also found in strains from different countries. A higher number of resistance genes was identified in strains from Mexico and other Latin American countries compared to strains from Europe and Asia. The results suggest that there is a high prevalence of hypervirulent strains in Mexico and Latin America and a higher frequency of antibiotic resistance genes compared to other regions of the world.

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128 - Increased Prevalence of Multi-Strain and Binary Toxin Positive *Clostridioides difficile* Infection in Clinical Isolates

Mônica Josiane Rodrigues-Jesus[1], Deiziane Viana da Silva Costa[1], Jae Hyun Shin[1], Cirle Alcântara Warren[1]

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Clostridioides difficile (*C. difficile*) continues to be the leading cause of antibiotic-associated diarrhea worldwide. Recurrent disease may be secondary to relapse from the primary infecting strain or reinfection from a newly acquired strain. Here, we evaluated the presence of multiple strains of *C. difficile* in human fecal specimens from patients diagnosed with CDI at UVA hospital from April/2021 to February/2022. We collected 174 fecal samples known to be all positive for *C. difficile* toxin B gene (*tcdB*). Three colonies were isolated from each specimen. Bacterial DNA was extracted to perform PCR assay (*tcdA*, *tcdB*, *cdtA and cdtB*). Comparing the genotypes of the three isolates obtained from each 174 fecal specimens, we observed that 34.5% (60/174) were discordant, suggesting the presence of multi-strains of *C. difficile*. Seven different genotypes were observed among the 522 clinical isolates and the most prevalent were $tcdA^{+}tcbB^{+}cdtA^{+}cdtB^{+}$ (50.19%), $tcdA^{+}tcbB^{+}cdtA^{+}cdtB^{-}$ (34.10%) and $tcdA^{+}tcbB^{+}cdtA^{-}cdtB^{-}$ (*13.02%*). Among the samples with single and multi-strains, 61.4% (70/114) and 58.3% (35/60) had strains positive for $cdtA^{+}cdtB^{+}$ genes respectively. Preliminary analysis of available clinical data in 57 patients (single strain=40 and multi-strain=17) showed that the presence of single strain is strongly associated with binary toxin positivity (p=0.0006) while most of the recurrent (41.2% p=0.43) and non-severe (82.4% p=0.07) cases were positive for multi-strains. These results indicate a high prevalence of multiple infection and presence of binary toxin positive strains in clinical cases of CDI. A trend in non-severe and recurrent CDI on the multi-strain cases suggest the potential relevance of detecting multi-strain infection.

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129 - Prevalence of *Clostridioides difficile* infection in hospitalized paediatric patients from a tertiary care hospital in Malaysia.

Mohammad Zahirul Hoque[1], Zulina Mazlan[2], Nur Nashyiroh[1], Khurshida Begum[3], M. Jahangir Alam[3]

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Background: Clostridioides difficile is a type of Gram-positive, anaerobic, spore-forming bacterium that causes infectious diarrhoea in patients receiving antibiotics. Clostridioides difficile Infection (CDI) has become a significant global public health concern, particularly in healthcare settings. **Objectives:** While there have been some reports on the prevalence of C. difficile infections in Malaysia and other Asian countries, our study aimed to detect and characterize C. difficile in hospitalized children from a large hospital in Sabah, Malaysia. **Methodology:** In this study, we examined a total of 473 stool samples collected from paediatric patients at Sabah Women & Children Hospital in Kota Kinabalu, Sabah, between August 2021 and June 2023. CDI was confirmed using a commercial test kit called C. DIFF QUIK CHEK COMPLETE (Alere, Techlab, USA), which detects both the glutamate dehydrogenase (GDH) antigen and toxins A & B of C. difficile in faecal samples. Data analysis was performed using Microsoft Excel and SPSS version 27.0 (IBM, USA). **Results:** Among the analysed stool samples, 120 out of 473 tested positive for either C. difficile antigen or toxin. The overall prevalence of C. difficile infection or colonization was found to be 25.3% (120/473). Specifically, 22.4% (106/473) of samples were positive for the antigen, while 3.0% (14/473) tested positive for the toxin. It is noteworthy that most of the positive cases were colonized with non-toxigenic C. difficile strains. **Conclusions:** Our study identified a low incidence of toxigenic C. difficile in the studied population. Long-term surveillance is essential to better understand the epidemiology of this emerging healthcare-associated infection.

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137 - Development of a next generation vaccine against *Clostridioides difficile* Infection

Mayuresh Abhyankar[1], Farha Naz[1], Feifan Xu[1], Joel Herbein[2], Christopher Fox[3], William Petri[1]

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Background. Mortality and high recurrence rates are the two pressing reasons to urgently develop a vaccine against *Clostridioides difficile* infection (CDI). Amongst the toxins produced by pathogenic *C. difficile*, TcdB toxin can undergo an accelerated evolution and appears to be the major virulence factor. Recent clinical trials by Pfizer and Sanofi Pasteur using alum adjuvanted inactivated toxins were terminated as none offered significant protection. We have established the use of a pharmaceutically-acceptable, dual Toll-Like Receptor agonist containing liposomal formulation (LS) to generate a sustained, concurrent, mucosal and systemic protective immune response against other priority pathogens. An ideal vaccine would neutralize and restrict CDI at the primary infection site, elicit a broad and high titer humoral response, generate long-lived T and B cell responses, and possess therapeutic potential to prevent R-CDI across all the ages.

Hypothesis. We hypothesize that a mucosal and systemic TcdB neutralizing antibody response will prevent primary and recurrent CDI.

Results. LS but not alum adjuvanted inactivated TcdB vaccine protected mice against weight loss upon challenge ($p=0.02$ on day 2, $p=0.006$ on day 3) and elicited mucosal IgA ($p=0.0004$) as well as neutralizing serum IgG responses (<0.0001). Interestingly, LS also favored generation of plasma IgG subtypes ($p<0.0001$) and intranasal administration appeared to improve durability of response ($p<0.001$ for B_{mem}). Overall, the dual TLR ligand liposome adjuvant showed promise in generating a balanced serum and mucosal antibody response. Additionally, its suitability for mucosal immunization offers a unique option of vaccination via multiple routes for an optimal protective response.

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148 - The effects of diet on rodent models of *Clostridioides difficile* infection

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Clostridioides difficile infection (CDI) is responsible for the majority of antibiotic-associated diarrhea, a potentially lethal outcome. CDI can result from the disruption of the resident gut microbiota. This can be brought on by the use of broad-spectrum antibiotics. Similarly, Western diets and popular weight-loss diets also drive large changes in the gut microbiome; however, there is conflicting literature on the effects of diets on CDI. In this study, we used antibiotic-induced murine and hamster CDI models to assess disease outcomes and microbial community dynamics on hypervirulent strain R20291-challenged animals fed various types of diets. In the murine CDI model, mice were fed either a high-fat/high-protein diet, a high-fat/low-protein diet, a high-carbohydrate diet, or a standard rodent diet. A high-fat/high-protein, Atkins-like diet led to severe CDI signs and 100% mortality. A high-fat/low-protein, medium-chain triglyceride (MCT)-like diet induced highly variable CDI outcomes. In contrast to the high-fat diets, mice fed a high-carbohydrate diet were protected from CDI, despite high refined carbohydrate and low fiber content. Diet and/or pre-spore-challenge antibiotic treatment decreased the abundance of Lachnospiraceae and Ruminococcaceae which may compete with C. difficile for amino acids and protect healthy animals from CDI in the absence of antibiotics. Conversely, in the hamster CDI model, a high-carbohydrate diet promoted dysbiosis, C. difficile carriage, and increased mortality. Together, these data suggest that antibiotic treatment intensified by high-fat/high-protein diet in mice exacerbates CDI while a high-carbohydrate protects from primary CDI; however, diets high in carbohydrates might paradoxically increase chances of CDI relapse in hamsters.

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POSTER SESSION II

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3 - Determining the effects of YabG alleles on the cleavage of *C. difficile* SleC

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[1]Texas A&M University

Clostridioides difficile forms metabolically dormant endospores, which allow for the dissemination from infected persons as well as resistance to harsh environments. Upon ingestion by the host, spores germinate into actively growing, toxin-producing vegetative cells. *C. difficile* spore germination is triggered in response to certain bile acids and amino acids e.g., taurocholic acid (TA) and glycine. In prior work that identified CspA as the co-germinant receptor, we identified mutation in the promoter or coding region of YabG. YabG is a sporulation-specific protease that processes preproSleC into proSleC and CspBA to CspB and CspA. To understand how the identified YabG alleles contribute to *C. difficile* spore germination, we introduced these mutations into an isogenic background. Building upon this, we sought to understand how YabG processes its targets. Spores derived from *C. difficile* Δ*yabG*, *C. difficile* *yabG*$_{C207A}$ (catalytically inactive), *C. difficile* *yabG*$_{A46D}$, *C. difficile* *yabG*$_{G37E,}$ and *C. difficile* *yabG*$_{P153L}$ strains germinate in response to TA alone and do not require co-germinants. Recombinantly expressed and purified preproSleC incubated with *E. coli* lysate expressing wild type YabG resulted in the removal of the pre sequence from preproSleC. Of the *yabG* mutants generated, interestingly only *yabG*$_{A46D}$ showed any catalytic activity towards preproSleC. Moreover, mutation of the YabG processing site in preproSleC (R119A) led to YabG shifting its processing to R115 or R112. Further investigation is ongoing to determine the effects of the identified mutations in the *yabG* promoter on gene expression.

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6 - Carriage of three plasmids in a single human clinical isolate of *Clostridioides difficile* and identification of a replicon from two of those

Anna M. Roseboom[1], Quinten R. Ducarmon[1], Bastian V.H. Hornung[1], Céline Harmanus[1], Monique J.T. Crobach[1], Ed J. Kuijper[1], Rolf H.A.M. Vossen[1], Susan L. Kloet[1], Wiep Klaas Smits[1]

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A subset of clinical isolates of *Clostridioides difficile* contains one or more plasmids and these plasmids can harbor virulence and antimicrobial resistance determinants (1). Despite their potential importance, *C. difficile* plasmids remain poorly characterized (2). We provide the complete genome sequence of a human clinical isolate (JMR5) that carries three high-copy number plasmids (pJMR5-1, pJMR5-4 and pJMR5-W) from three different plasmid families that are therefore compatible (3). For two of these, we identify a region capable of sustaining plasmid replication in *C. difficile* that is also compatible with the plasmid pCD630 that is found in many laboratory strains. Together, our data advance our understanding of *C. difficile* plasmid biology.

1. Hornung *et al*. Microb Genom. 2019 Sep;5(9):e000296. doi: 10.1099/mgen.0.000296.

2. Smits *et al*. Curr Opin Microbiol. 2022 Feb;65:87-94. doi: 10.1016/j.mib.2021.10.016.

3. Roseboom *et al*. Plasmid. 2023; 125:102669. doi: 10.1016/j.plasmid.2022.102669.

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19 - Phenotypic Characterization of *C. difficile* Ribotypes

Merilyn Beebe[1], Joseph Sorg[1]

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In recent years, the CDC has reported that *Clostridioides difficile* infections (CDI) have caused almost 300,000 hospitalizations per year. Of these, approximately 15-30% are the result of recurring infections. The prevalence and persistence of CDI in hospital settings has resulted in extensive collection of *C. difficile* clinical isolates and their classification, typically by ribotype. While much of the current literature focuses on one or two prominent ribotypes (e.g. 027), recent years have seen several new ribotypes dominate the clinical landscape. Currently, no single phenotype or set of phenotypes have been identified to indicate why certain ribotypes are more prominent, or harmful, than others. We seek to identify such distinguishing characteristics by creating a mini-library compromised of clinical isolates encompassing various ribotypes spanning each known *C. difficile* clade. Members of the library are assayed for growth, sporulation, germination, bile acid sensitivity, and bile salt hydrolase activity. In viewing these phenotypes through the ribotype lens, we hope to identify a set of physiological traits that distinguish each ribotype and explain their clinical behavior. In addition to aiding in the study of *C. difficile*, identification of these traits would improve the treatment of CDI by allowing for the personalization of treatments based on strain/ribotype.

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21 - Prevalence and characterization of *Clostridioides difficile* in dogs attended at veterinary clinics in Rio de Janeiro

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Clostridioides difficile may represent a community pathogen, and domestic dogs can be a potential source of exposure. Thus, the goal of this study is to confirm the presence of *C. difficile* in domestic dogs examined at veterinary clinics in Rio de Janeiro and to correlate any findings with the clinical parameters of those animals. Ninety-three stool samples were cultured in selective medium (*Clostridioides difficile* Brucella agar - CDBA) and isolates were characterized by ribotyping, toxin genes *(cdt*b, *tcd*A and *tcd*B) by PCR and antibiotic susceptibility test (vancomycin [VAN], metronidazole [MET], erythromycin [ERY], rifampicin [RIF] and moxifloxacin [MOX]). In order to better investigate the epidemiology of the infection in the species, an epidemiological questionnaire was delivered to the dog's tutors. *C. difficile* was isolated from 12.9% (12/93) with 83,3% (10/12) of the positive animals presenting diarrhea and 58.3% (7/12) under antibiotics use at least for 3 months, before stools were collected. PCR-Ribotyping revealed that 75% (9/12) of *C. difficile* strains belonged to RT106 (toxigenic), 16,6% (2/12) to RT014/20 (toxigenic) and 8,3% (1/12) to RT010 (non toxigenic). In total, 17% (2/12) of strains were resistant to VAN, MET and RIF, while 33,3% (4/12) to ERY. Resistance to MTZ is probably related to the presence of the pCDMETRO plasmid and is under investigation. The relevance of *C. difficile* in domestic animals requires additional research to better understand the epidemiology of this pathogen in the community.

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24 - The small acid-soluble proteins of *Clostridioides difficile* regulate sporulation in a SpoIVB2-dependent manner.

Hailee Nerber[1], Marko Baloh[1], Joseph Sorg [1]

[1]Texas A&M University

Spores are metabolically dormant forms of bacteria that survive desiccation, extreme temperatures, and chemical and UV exposure. The overall structure of endospores is conserved among spore-forming bacteria. Inside, the core contains DNA, RNA, dipicolinic acid, and proteins, such as the small acid-soluble proteins (SASPs). In *B. subtilis,* the primary SASPs, SspA and SspB, protect the DNA from UV damage by coating the DNA and altering the DNA conformation to discourage cis-syn thymine dimer formation. *C. difficile* encodes *sspA* and *sspB* orthologues. Though the *C. difficile* SASPs functioned as predicted in UV resistance, we surprisingly found that the combined deletion of *sspA* and *sspB* prevented spore formation, a novel phenotype. This suggests a possible regulatory role of SspA and SspB during sporulation. Using an ethyl methanesulfonate (EMS) mutagenesis selection strategy, we identified mutations in *CDR20291_0714* (*spoIVB2*) that suppressed the immature spore formation phenotype. SpoIVB2 is a predicted peptidase that is homologous to *B. subtilis* SpoIVB, a protein involved in the signaling cascade for sporulation. We hypothesize that SASP binding influences transcript levels of *spoIVB2,* regulating the levels of SpoIVB2 present in the forespore. When SASPs are not present, SpoIVB2 accumulates aberrantly and interferes with the sporulation process through an unknown mechanism. Investigations are ongoing to determine the pathway in which SspA, SspB, and SpoIVB2 interact to influence sporulation in *C. difficile.*

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28 - The role of polyamines in *Clostridioides difficile* pathogenesis

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Background: Despite its anaerobic nature, *Clostridioides difficile* (*Cd*) can survive in low levels of oxygen throughout the human intestinal tract. In facultative anaerobes such as *Escherichia coli*, the oxidative stress response is dominated by antioxidant defense enzymes and transcriptionally upregulated by cellular polyamines, which are organic compounds containing multiple amine groups that play vital roles in cell survival and growth.

Purpose: Understanding the molecular underpinnings of the oxidative stress response in *Cd* is crucial for characterizing its pathogenesis in the low-oxygen environment of the human gut and subsequently informing therapeutics to treat initial and recurring CDI.

Methodology: Candidate genes involved in oxidative stress response and polyamine synthesis/uptake were deleted from the *Cd630* genome, including those encoding a polyamine transporter (*CD630_RS05720-RS05735*), spermidine synthesis enzymes (*CD630_RS05025-RS05040*), and reaction oxygen species scavengers. Mutants will be grown in anaerobic or low oxygen (2%) conditions, with or without putrescine supplementation, and examined during exponential growth phase. Cellular polyamine levels of mutants and wild-type *Cd630* will be measured using the BioVision Total Polyamine Assay Kit.

Results: While *Cd* growth is diminished at 2% oxygen relative to anaerobic growth in standard media, growth at low oxygen is substantially greater than in anaerobic conditions when supplemented with putrescine.

-**Conclusion**: This preliminary data suggests a polyamine-mediated protective mechanism against oxidative stress. Future experiments described above will establish the mechanistic roles of the candidate gene products and elucidate the connection between polyamines and oxygen tolerance in *Cd*, informing our limited understanding of its pathogenesis of the human gut.

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31 - *Clostridioides difficile* infection perturbs colonic structure and slows gut transit

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The gastrointestinal tract (GIT) is responsible for essential digestive functions, which rely on tight regulation by a network of neurons and glial cells embedded in the gut wall, termed the enteric nervous system (ENS). These cells direct numerous gastrointestinal motor, immune and homeostatic functions, which are vital for normal gut activity. A critical ENS-mediated function is the coordination of gastrointestinal smooth muscle to generate force for the transit of intestinal contents through the gut. Consequently, ENS dysregulation and functional dysmotility underlie various gastrointestinal disorders. However, whether ENS dysregulation and altered gut motility contribute to enteric infection has not been as greatly explored. Therefore, we aimed to determine if infection with *Clostridioides difficile*, a pathogen that causes extensive colonic damage, affects the ENS and explore how ENS dysregulation contributes to disease. Using a mouse model of *C. difficile* infection (CDI), we demonstrated that severe disease perturbed smooth muscle and ENS architecture. To link these structural changes to functional motor alteration, we showed that CDI decreased the contractive ability of the colon, resulting in a slowing of fecal transit through the GIT. As diarrhea, which is defined as 3 or more loose stools per day, is a defining characteristic of CDI, patients who do not exhibit an increased frequency of stool output may not receive timely diagnosis or treatment. Therefore, this work will aid in improving CDI diagnostic criteria to ameliorate the clinical outcomes of patients who present with atypical manifestations of CDI.

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34 - *Clostridioides difficile* uses glutathione as a sulfur source

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Several enteric pathogens, including *Clostridioides difficile,* induce host inflammation to gain a metabolic advantage over the gut microbiota so that they can establish and maintain a niche. *C. difficile*, like other Clostridia, ferments amino acids via Stickland fermentation for energy generation. An unexplored source of amino acids that *C. difficile* encounters during infection is glutathione (GSH). GSH is a tripeptide (Glu-Cys-Gly) and the most abundant low-molecular-weight thiol in mammalian cells. Because phylogenetically diverse *C. difficile* strains contain homologs of GSH utilization genes characterized in other pathogens, we hypothesized that *C. difficile* metabolizes GSH. Using *in vitro* growth curves, we showed that GSH enhances *C. difficile* growth. In addition, RNA-seq showed that GSH-exposed *C. difficile* upregulates genes involved in sulfur metabolism. Taken together, these data suggests that *C. difficile* utilizes GSH as a source of cysteine which is subsequently funneled into sulfur metabolism. Our ongoing work seeks to determine how *C. difficile* accesses GSH in a murine model of infection. Overall, this work provides an important example of the metabolic versatility of *C. difficile* and serves as the foundation for continued investigation into the ways it can capitalize on available nutrients during infection.

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37 - Inhibition of *Clostridium perfringens* Spore Growth by Synergistic Effects of Chitosan and Nisin

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Clostridium perfringens is a spore-forming bacterium and a major causative agent of *C. perfringens* food poisoning (FP). Due to high heat-resistance of spores, it is of great intertest to develop strategies alternative to thermal processing to inactivate or eliminate *C. perfringens* spores from food products. Our previous studies showed that only a high concentration of chitosan (0.2%) can effectively inhibit the growth of *C. perfringens* spores in cooked-chicken meat. However, nisin even at a very high concentration (5%) was unable to inhibit *C. perfringens* spore growth in cooked-chicken meat. As some recent studies showed that nisin is effective in combination with other preservatives, in this study we evaluated the inhibitory effects of chitosan-nisin combination against germination, outgrowth and vegetative growth of spores of *C. perfringens* in laboratory medium and chicken meat. Among many tested concentration combinations of chitosan-nisin, the most effective ($p<0.01$) inhibition of spore germination and outgrowth in laboratory medium was observed with a mixture of 0.025% chitosan and 0.075% nisin. Furthermore, the chitosan-nisin combination (0.025% each) also illustrated significant ($p<0.01$) inhibition in vegetative growth of spores of *C. perfringens*. However, a little-bit higher concentration combination of chitosan-nisin (0.01% each) was needed to effectively inhibit *C. perfringens* spore growth in cooked-chicken meat. Collectively, our results should contribute to establish an effective use of chitosan-nisin combination in meat products to control *C. perfringens*-associated diseases.

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41 - Microbiome and Health-Related Quality-of-Life Shifts in Patients With Clostridioides difficile Infection After Treatment With Fecal Microbiota, live-jslm

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Introduction: Microbiota-based treatments have shown efficacy in reducing recurrent *Clostridioides difficile* infection (rCDI), but data on patient health-related quality-of-life (HRQOL) impacts are limited. Fecal microbiota, live-jslm (REBYOTA™, abbreviated here as RBL, previously known as RBX2660), is the first single-dose, rectally administered, microbiota-based live biotherapeutic approved by the US Food and Drug Administration to prevent rCDI in individuals ≥18 years old following antibiotic treatment for rCDI. Here, HRQOL results of RBL-treated participants in a randomized, double-blinded, placebo-controlled phase 3 trial PUNCH CD3 (NCT03244644) are reported.

Methods: Participants ≥18 years old with ≥ 1 rCDI episode were treated with a single dose of RBL or placebo. The *Clostridioides difficile* Quality of Life Survey (Cdiff32), a self-assessed, CDI-specific questionnaire on physical, mental, and social health, evaluated participants 8 weeks after treatment.

Results: The differences between RBL and placebo in the Cdiff32 total and mental scores after 8 weeks were 6.11 (95% CI: [0.14-12.08], $p<0.05$) and 7.07, (95% CI: [0.28-13.86], $p<0.05$), respectively, indicating sustained HRQOL improvements versus placebo. These improvements correlated with increased relative abundance of Bacteroidia and Clostridia and decreased relative abundance of Gammaproteobacteria and Bacilli. The secondary to primary bile acid (BA) ratio was also increased in RBL responders compared with RBL non-responders and placebo responders and non-responders.

Discussion: In RBL-treated participants, a restoration of the microbiome and BA profiles was observed with increases in the HRQOL. The microbiota-gut-brain axis is posited to modulate these effects through immune, gastrointestinal, and central nervous system functions.

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43 - Characterization of a sequence invariable region (SIR) in *Clostridioides difficile* TcdB

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Several distinct subtypes of *Clostridioides difficile* TcdB have been identified in recent years. Correlating sequence and functional differences in these variants of TcdB has led to the discovery of specific amino acids involved in receptor tropism and cell entry. Here, we predicted that, in contrast to the variable sequences, highly conserved sequences have been evolutionarily preserved due to their role in indispensable activities shared among all the variants of TcdB. Exploring these regions, which have not previously been associated with known functions of TcdB, can provide new insights. The longest stretch of conserved sequence is found at the carboxy terminus of TcdB where 76 amino acids spanning residues 2291-2366 are 100% conserved between reference sequences from the TcdB1-4 subtypes. To identify a functional role for this sequence invariable region (SIR), the last 228 bp of *tcdB2* were deleted in the epidemic *C. difficile* R20291 strain which resulted in a truncated TcdB2 lacking residues 2291-2366 (hereafter *C. difficile* TcdB2$_{\Delta 2291\text{-}2366}$). In the absence of the SIR domain, TcdB2$_{\Delta 2291\text{-}2366}$ retained cytotoxic activity but, unlike wildtype TcdB2, was not released from cells into the extracellular environment even in growth conditions that favored autolysis. Collectively, our data demonstrates that the SIR domain is necessary for proper release of the toxin from cells and ongoing mechanistic studies will provide new details into how this large toxin escapes from *C. difficile*.

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45 - Phase variation-dependent localization of *Clostridioides difficile* variants during infection

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Phase variation-dependent expression of flagellum and toxin genes in *Clostridioides difficile* is mediated by the invertible "*flg* switch," a 154 bp DNA sequence upstream of the *flgB* operon whose orientation impacts flagellar gene expression in an ON/OFF manner. Consequently, *C. difficile* exists as a phenotypically heterogeneous population of toxigenic, flagellated cells and aflagellate cells attenuated for toxin production; the proportion of each phenotypic variant in a population is influenced by environmental stresses. We hypothesize that selective pressures encountered during infection alter the phenotypic composition and biogeography of the *C. difficile* population. We examined the spatiotemporal distribution of *C. difficile* in the intestinal tract using a mouse model of CDI. Mice were infected with wildtype R20291 with the *flg* switch 93% in the ON orientation, and we used qPCR to assess changes in the proportion of ON/OFF cells over time and in different segments of the intestine. The proportion of *flg*-OFF cells in the population increased on day 2, then the proportion of *flg*-ON cells recovered on day 5. These results suggest aflagellate and nontoxigenic cells are more fit at the peak of infection, whereas cells that produce toxins and flagella are more advantageous later in infection. We also found that the population in fecal shedding shifts to the OFF orientation over time, suggesting aflagellate and nontoxigenic cells are shed during infection. These results show how the host environment influences the population of *C. difficile* phase variants, and future work will more precisely localize phenotypic variants in the intestinal tract.

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47 - A novel medium for cultivating murine gut microbiota

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Commensal gut microbiota is crucial for protecting the host against enteric pathogens including *Clostridioides difficile* (*C. difficile*), a major cause of healthcare-associated infections. However, studying the role of specific gut microbes in mouse models of *C. difficile* infection (CDI) can be challenging due to difficulties in culturing most murine gut microbes. Here, we used comparative fecal metabolomics to guide the design of media to facilitate cultivation of novel murine microbes. We colonized germ-free (GF) C57BL/6 mice with serial dilutions of conventional murine fecal microbiota, and then challenged with *C. difficile* spores. Mice harboring higher dilutions of fecal microbiota succumbed to CDI, whereas mice with lower dilutions were resistance to *C. difficile* challenge. We then compared fecal samples from *C. difficile* susceptible and resistance mice using targeted and untargeted metabolomics. Fecal metabolites from *C. difficile*-resistant mice were enriched in glucose but depleted in sucrose, trehalose, sorbitol, raffinose, lactose, and mannitol, suggesting that *C. difficile* resistant microbiota preferentially utilized a variety of carbon source other than glucose. Using this information, we designed a novel growth medium and successfully cultured 22 unique murine gut microbes belonging to the *Firmicutes* phylum, including 18 that were not previously cultured or reported. Comparative fecal metabolomics may be a promising approach for designing novel culture media that enhance the cultivability of murine gut microbes relevant in *C. difficile* pathogenesis.

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49 - Redistribution of the Novel *Clostridioides difficile* Spore Adherence Receptor E-Cadherin by TcdA and TcdB Increases Spore Binding to Adherens Junctions

Pablo Castro-Córdova[1], Macarena Otto-Medina [1], Nicolás Montes-Bravo[1], Christian Brito-Silva[1], D Borden Lacy[2], Daniel Paredes-Sabja[1, 3]

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Clostridioides difficile causes antibiotic-associated diseases in humans, ranging from mild diarrhea to severe pseudomembranous colitis and death. A major clinical challenge is the prevention of disease recurrence, which affects nearly ~20 to 30% of the patients with a primary C. difficile infection (CDI). During CDI, C. difficile forms metabolically dormant spores that are essential for recurrence of CDI (R-CDI). In prior studies, we have shown that C. difficile spores internalize into intestinal epithelial cells (IECs), and that intracellular spores contribute to R-CDI. However, this interaction remains poorly understood. In this work, we explored how *C. difficile* spores interacts with IECs at various differentiation stages, and the impact of TcdA/TcdB intoxication in spore adherence and internalization. As expected, TcdA and TcdB lead to adherens junctions opening and to increased spore adherence to IECs. Confocal micrographs demonstrate that *C. difficile* spores associate with accessible E-cadherin, which was increased in TcdA/TcdB intoxicated IECs. Blockage of accessible E-cadherin with anti-E-cadherin antibodies, decreased spore adherence and entry into IECs. By enzyme-linked immunosorbent assay (ELISA), immunofluorescence, and immunogold labeling, we observed that E-cadherin binds to C. difficile spores, specifically to the hairlike projections of the spore. Overall, these results demonstrate that E-cadherin acts as a spore adherence receptor to IECs and reveal that TcdA/TcdB-mediated increase in spore adherence to IECs is E-cadherin dependent. These observations expand our knowledge of how *C. difficile* spores interact with host cells

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51 - The inhibitory effects of zinc on autolysin activity and cell lysis of *Clostridium perfringens*

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Clostridium perfringens is an obligate anaerobe associated with food poisoning and gas gangrene. Acp, one of peptidoglycan hydrolases (PGHs) of *C. perfringens*, has been identified as a key player in regulating cell division. Acp-defective cells (strain 13 *acp::erm*) are unable to divide normally, and their morphology of cells is observed as a long chain [1]. This report suggested that Acp is required in the vegetative growth of *C. perfringens* cells. On the other hand, dysregulated activation of Acp under oxidative stress or exposure to bile salt leads to detrimental cell lysis. Therefore, the experimental handling of *C. perfringens* cells is needed for caution under normal atmospheric conditions.

We investigated the influence of various metals on Acp activity using renaturing SDS-PAGEs (zymography), revealing the inhibitory effects of zinc on Acp activity. Our findings demonstrate that zinc at 0.25-10 mM exerts a significant reduction in cell lysis compared to the control under a normal atmosphere. Notably, 5-10 mM zinc concentrations achieve over 90% suppression of cell lysis. Interestingly, in GAM medium pre-supplemented with zinc (5-10 mM zinc-added), the cell lengths were significantly increased in each group relative to the control. These results elucidate the inhibitory effects of zinc on Acp activity within *C. perfringens*, resulting in diminished cell lysis under normal atmospheric conditions.

Understanding the molecular mechanisms underlying Acp regulation and the impact of zinc-mediated modulation could provide valuable insights for the experimental handling of *C. perfringens* cells.

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53 - Mechanisms and consequences of intestinal barrier disruption and solute transport dysregulation during *Clostridioides difficile* infection

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Clostridioides difficile infection (CDI) is the leading cause of hospital-acquired diarrhea and pseudomembranous colitis, but the pathophysiology of CDI-induced diarrhea remains incompletely understood. In this study, we investigated changes in solute transport and intestinal barrier integrity during acute CDI. Mice were inoculated with *C. difficile* spores, and we assessed intestinal permeability and electrogenic ion transport at two days post-infection. Our findings revealed that intestinal permeability is increased through a size-selective, tight junction-dependent pathway, without significantly altering transmucosal resistance. Unlike other infectious diarrheal conditions, CDI did not induce Cl^- hypersecretion or affect solute absorption through Epithelial Na^+ Channels. Instead, we observed a significant loss of function of Na^+-Glucose Co-transporter 1 (SGLT1) accompanied by increased glucose concentrations in stool. Furthermore, we discovered that SGLT1, along with two previously implicated ion transporters, Na^+/H^+ Exchanger 3 (NHE3) and Downregulated in Adenoma (DRA), were downregulated at the transcript level during acute CDI. Notably, the absence of one of these transporters alone in humans can cause specific types of congenital diarrhea, emphasizing their crucial role in solute absorption. In conclusion, our study highlights the significance of both toxins A and B in inducing the pathophysiological changes responsible for diarrhea during acute CDI. We demonstrate that increased intestinal permeability and impaired water, Na^+, Cl^-, and carbohydrate absorption mediated by SGLT1, NHE3, and DRA contribute to diarrhea. Ongoing research aims to uncover the mechanisms of dysregulation of epithelial ion transporters and tight junctions, and to investigate the effects of luminal solute imbalances on *C. difficile* pathogenesis.

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55 - The *Clostridioides difficile* cell wall enigma: Evidence for a novel peptidoglycan crosslinking enzyme

Kevin Bollinger[1], Ute Müh[1], Karl Ocius[2], Alexis Apostolos[2], Richard Helm[3], Marcos Pires[2], David Popham[4], Craig Ellermeier[1], David Weiss[1]

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The cell wall is an essential structure for bacteria and contains strands of peptidoglycan (PG) which are formed by a repeating disaccharide subunit of N-acetyl muramic acid and N-acetyl glucosamine. PG is crosslinked together by a peptide sidechain attached to the N-acetyl muramic acid. There are two main forms of PG crosslinking in bacteria, 4-3 crosslinking made by penicillin binding proteins (PBPs) and 3-3 crosslinking formed by L,D-transpeptidases (Ldts). The majority of PG crosslinks in most bacteria are 4-3 crosslinks with a small proportion of 3-3 crosslinks. In contrast, in *C. difficile* over 70% of the dipeptide crosslinks are 3-3 crosslinks. *C. difficile* has 3 annotated Ldts. Using CRISPR mutagenesis, we successfully deleted all known *ldt*s (*Δldt1-3*). The deletions were confirmed using PCR, whole-genome sequencing, and western blot. The *Δldt1-3* mutant has no growth defect, no morphological change, nor any change in antibiotic sensitivity. Using a fluorescent tetrapeptide substrate that is specifically incorporated by Ldts, we see a large reduction in Ldt activity in our *Δldt1-3* mutant. However, there is still fluorescent tetrapeptide incorporation occurring in the *Δldt1-3* mutant, despite no known Ldts being present. Furthermore, we observe increased incorporation of the tetrapeptide label at the midcell in both WT and *Δldt1-3* mutant. Shockingly, muropeptide analysis revealed no change in 3-3 crosslink levels in the *Δldt1-3* mutant compared to WT. These data indicate there is an unknown protein, or proteins, in *C. difficile* responsible for making the majority of the 3-3 crosslinks.

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58 - Unravelling the interaction specificity of *Clostridioides difficile* bacteriophages

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Phage therapy, using bacteriophages (or phages), which are bacterial viruses that specifically kill bacteria, is gaining attention as a promising solution against antibiotic resistance. Broad-spectrum antibiotics often result in microbiota disbalance due to off-target effects and contribute to issues such as *Clostridioides difficile* (CD) infections with frequent relapses. Phages, with their high host specificity, offer an alternative as a targeted therapy. However, a thorough understanding of CD-phage interactions is crucial for successful infection control. Our research has revealed that the surface layer protein, SlpA, serves as a receptor for CD phages. Moreover, we observed that different SlpA isoforms are involved in the specificity of interaction with them. Also, we identified the D2 domain of the SlpA protein, as important for infection by certain siphophages (Royer *et al*, Microbiol. Spectr. 2023). Recent results have shown that D2 is also needed for infection by certain myophages. We are currently creating a collection of mutated SlpA to gain deeper insights into which regions of the protein are involved in the interaction with various phages. Bioinformatics approaches are used to predict the *in silico* structures of SlpA-interacting phage receptor-binding proteins, demonstrating the structural diversity of these proteins. Ultimately, our findings will contribute to the design of recombinant phage cocktails targeting different SlpA isoforms to enhance the phage host range and pave the way for future applications of phage therapy against CD infections.

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63 - Six human bacteria consortium suppresses *C. difficile* growth and toxin production independent of secondary bile acid inhibition in a humanized mouse model

James Martin[1], Daniel Marquina[2], Gurjit Sidhu[3], Alexandra Peterson[2], Joan Whitlock[2], Preethi Sudhakara[1], Gary Wang[4]

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Clostridioides difficile infection (CDI) is the primary cause of antibiotic-associated diarrhea and a significant cause of morbidity and mortality in healthcare settings. Firmicutes are commonly depleted in patients with CDI and their recovery has been associated with clinical cure. However, the identity of commensal Firmicutes bacteria that correlates with CDI protection remains poorly understood. We hypothesized that a defined consortium of Firmicutes isolated from healthy human volunteers could confer resistance against *C. difficile* challenge in a germ-free mouse model. We cultured 100 spore-forming bacteria from humanized mice resistant to *C. difficile* challenge, then screened and constructed a defined consortium containing six spore-forming Firmicutes bacteria. We showed that germ-free mice could be stably colonized with the six-isolate consortium, and that challenge with *C. difficile* spores resulted in a clinically asymptomatic phenotype which was indistinguishable from healthy controls. In contrast, germ-free mice died within 48 hours of *C. difficile* challenge. Addition of six-isolate consortium to *C. difficile* susceptible mice was sufficient to rescue and protect the mice from *C. difficile* challenge. Bile acid analysis of fecal metabolites showed an absence of secondary bile acids in the 6-isolate mice, suggesting a bile acid-independent mechanism of protection. Importantly, mice colonized with the 6-isolate consortium had significantly lower *C. difficile* growth and *C. difficile* toxin levels compared to mice that developed diarrhea or died from *C. difficile* challenge. These results suggest that the 6-isolate consortium protects GF or susceptible mice from CDI by suppressing *C. difficile* growth and toxin production in a bile acid-independent manner.

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65 - The anchoring of the polysaccharide II is essential for *Clostridioides difficile* survival

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In this study, we investigated the role of the two *lcp* genes. We constructed single and double mutants of *lcp* genes. Surprisingly, we were unsuccessful to obtain the double mutant whereas the two single mutants were easily obtained by an allelic exchange technique. This suggests that *lcp* genes have redundant functions. To delete both *lcp* genes, we developed a conditional lethal mutant technique. The first step was to construct a strain containing a second copy of *lcpB* in a chosen region of the chromosome, expressed under the control of an anhydrotetracycline inducible promotor, pTet. Then, we replaced the ORFs by a resistance cassette in the native locus and so deleted both *lcpA* and *lcpB*. In this conditional mutant, we were able to modulate the expression of *lcpB*. Thanks to this tool and the production of highly specific anti-polysaccharide II antibodies, we highlighted the essentiality of the polysaccharide II anchoring. Using immunofluorescence microscopy, we showed that in single mutants, the polysaccharide II layer is abnormal in comparison to the wild-type strain. In the double mutant with low *lcpB* expression, we observed ellipsoid cells. Complementation with *lcpA* or *lcpB* restores the rod-shape morphology and the normal abundance of polysaccharide II. Additional results show a defect of the S-layer anchoring in the conditional mutant strain. In conclusion, our results show the critical role of polysaccharide II anchoring in growth, elongation, and correct surface set-up of *C. difficile.* Our technique provides new opportunities to study essential genes in *C. difficile*.

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67 - A new method for the production of TcdA and TcdB toxins from *Clostridioides difficile*

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Clostridioides difficile (CD) is a major pathogen responsible of digestive healthcare associated infections. Antibiotic treatments are the main risk factor for CD infections (CDI) because they disrupt the gut microbiota. During dysbiosis, CD spores germinate into vegetative forms which then colonize the gut microbiota, multiply, and produce virulence factors, especially toxin A (TcdA) and toxin B (TcdB). The action of these two toxins will cause an alteration of the cytoskeleton, neutrophils recruitment, thus promoting an inflammatory process, and leading to the clinical manifestations of CDI.

The study of toxins is a crucial step in exploring the virulence of this pathogen. Currently, the toxin purification process is either laborious and time-consuming in CD or performed in heterologous hosts. Thus, we propose an easy method to obtain rapidly functional toxins directly using CD as the host.

First, we generated two recombinant CD strains with a sequence encoding a His-tag at the 3'-end of the CD 630Δ*erm tcdA* and *tcdB* genes and then replaced the toxins native promoter with the Ptet promoter inducible by Anhydro-tetracycline (ATc). Then, using these strains, recombinant toxins A (rTcdA) and B (rTcdB) can be produced after induction with ATc and purified using a one-step Ni-affinity chromatography with a simple imidazole elution.

Recombinant toxins were compared to native toxins according to cytopathic activity using cell-cytotoxicity-neutralization assay on Vero-cells, and their use as antigens in ELISA assay using Human serums from cured-CDI patients.

In conclusion, construction of recombinant strains allowed us to rapidly produce biologically active rTcdA and rTcdB.

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70 - *Clostridioides difficile* increases undecaprenyl pyrophosphate recycling and drug efflux in response to iron starvation

Martin Douglass[1], Eric Skaar[1]

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Clostridioides difficile infection (CDI) is the leading nosocomial intestinal infection in the United States and an urgent threat to public health. CDI onset begins with C. difficile outcompeting both the host microbiota and the innate immune response for limited nutrients. A critical factor in the host immune response to CDI is the innate immune protein calprotectin (CP) that chelates essential nutrient metals from the pathogen through a process termed nutritional immunity. CP is essential for the host to combat CDI, yet how C. difficile overcomes CP to acquire nutrients is not well understood. To uncover how C. difficile responds to nutritional immunity, we evaluated the transcriptional changes that C. difficile undergoes when challenged with CP. We identified a putative two-component system (TCS), 2822 and 2823, to be transcriptionally increased in the presence of CP and iron chelators. Mutants lacking this TCS exhibit a growth defect in iron limiting conditions. Furthermore, we found 2822/2823 regulates three genes immediately downstream: 2821, 2820, and 2819. Based on bioinformatic predictions, 2820 and 2819 encode an ATP driven efflux pump, and 2821 encodes an undecaprenyl pyrophosphatase. Further experiments revealed that 2822/2823 is activated by the antibiotic bacitracin, and mutants lacking the TCS are extremely sensitive to the cell surface targeting molecules bacitracin and vancomycin, the latter of which is clinically relevant. Our results support a model in which C. difficile overcomes nutritional immunity by coordinating an increase in undecaprenyl pyrophosphate recycling and drug efflux to defend against external threats such as cell envelope targeting antimicrobials.

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73 - Understanding *Clostridioides difficile* RT023 xylitol utilization

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Several ribotypes of *Clostridioides difficile* have emerged over the last three decades, yet the factors driving emergence remain unclear. Based on whole genome sequencing, *Clostridioides difficile* strains have been grouped into five distinct clades. Recently there was an increase in infections caused by the rarest clade of *C. difficile*, clade 3 (RT023), which have been understudied compared to other *C. difficile* clades. We profiled the growth of 12 RT023 strains on 190 unique carbon sources and found they were able to grow on xylitol, a sugar alcohol commonly used as a food additive in humans and animals. All other ribotypes of *C. difficile* tested displayed little to no growth and were inhibited from growth in high concentrations of xylitol. Genome sequencing of these strains identified that RT023 strains acquired a gene with high similarity to xylitol dehydrogenase (*xdh*) in a mobile genetic element (MGE) that is missing in all other *C. difficile* ribotypes. We deleted the MGE in a strain of RT023 and observed poor growth on xylitol, indicating that the genes contained within the MGE are necessary for xylitol utilization. We performed competition assays in minibioreactor arrays and observed that RT023 strain outcompeted a RT027 strain in the presence of xylitol. Together, these data suggest that the *xdh* is beneficial for growth on xylitol and increases fitness compared to non-utilizing strains. This work will help us understand niche adaptation of RT023 strains and their increasing prevalence in hospitals.

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77 - *Paeniclostridium sordellii* NanS works synergistically with TcsL to increase cytotoxicity and the severity of pathogenesis in the murine uterus

Sarah Bernard[1], Heather Kroh[2], Kay Washington[2], Borden Lacy[1, 2]

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Paeniclostridium sordellii, a pathogen capable of causing lethal uterine infections post-partum or post-abortion, produces a variety of potential virulence factors. Lethal toxin (TcsL) has been found to be essential for lethal *P. sordellii* infections, but the physiological role of NanS, a sialidase, remains elusive. Here, we purify enzymatically active *P. sordellii* NanS and report the crystal structure. We show that NanS works in a synergistic manner with TcsL to increase cytotoxicity and cell rounding of tissue culture cells. Using a mouse model of hormone-dependent uterine intoxication, we show that NanS augments TcsL-induced lethality in diestrus mice. In estrus mice, the combination of NanS and TcsL significantly increases uterine histologic damage compared to challenge with TcsL alone. This suggests that NanS enhancement of TcsL-induced uterine epithelial injury may potentiate TcsL access to the tissue when mucus is present.

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79 - Metagenomic Evaluation of Ibezapolstat Compared to Other Anti-*Clostridioides difficile* Agents

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Background: Ibezapolstat (IBZ), a novel DNA polymerase IIIC inhibitor is currently in clinical trials for the treatment of *Clostridioides difficile* infection (CDI) in adults. IBZ causes less microbiome disruption than vancomycin in humans. No comparative microbiome studies exist for other anti-*C. difficile* antibiotics. The purpose of this study was to compare IBZ gut microbiome changes *in vivo* to other anti-*C. difficile* antibiotics.

Method: Germ-free (GF) mice (six per group) were randomly assigned to IBZ, fidaxomicin, vancomycin, metronidazole, or control. An oral gavage of healthy human-derived fecal slurry was given to GF mice and allowed to establish (14-days) followed by 14-days of antibiotics. Stool samples were collected at baseline and after 2 and 10 days of antibiotics to assess antibiotic microbiome effect (16S rRNA metagenomics).

Results: Prior to antibiotic initiation, Shannon's index alpha diversity was similar across treatment groups. There was a significant decrease in alpha diversity in all antibiotic groups compared to control ($p<0.05$). Beta diversity analysis showed distinct clustering in mice given IBZ versus all other antibiotics. At the phylum level, a significant proportional increase of Bacteroidetes and decrease in Verrucomicrobiota was observed in the IBZ group while the relative abundance of Proteobacteria increased in fidaxomicin, vancomycin and metronidazole groups.

Conclusion: IBZ given to human microbiome GF mice resulted in favorable and distinctive changes in the gut microbiome compared to fidaxomicin and the well-known gut microbiota disrupting agents, vancomycin and metronidazole. These results support the continued clinical development of IBZ for the treatment of CDI.

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82 - *Clostridium spiroforme* associated enteric disease in rabbits: retrospective study of 32 cases and literature review

Francisco Uzal[1], Laura Tuomisto[2], Arturo Oliver Guimerá[1], Isabel Casanova[1], Sari Kanfer[3], Kevin Keel[1], Javier Asin[1], Denise Imai[1], Fabio Mendonca[4]

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Clostridium spiroforme is responsible for spontaneous and antibiotic-associated enteric disease in rabbits. The disease is known as *C. spiroforme* associated enteric disease (CSAED), and it is clinically characterized by anorexia, diarrhea, or sudden death. *C. spiroforme* produces *C. spiroforme* toxin (CST), which is thought to be the main virulence factor. Diagnosis is usually based on gross and microscopic pathology, coupled with detection of the characteristic coiled bacteria in intestinal smears. Isolation of *C. spiroforme* is, however, often challenging. We reviewed 32 rabbits with diagnosis of CSAED submitted for necropsy to several diagnostic laboratories between 1992 and 2019. The most common gross findings reported were dilation of the cecum with brown to green, watery content (20/32), soiling of the perineum, tail and/or hind legs with diarrhea (16/32), serosal petechiae or ecchymoses in the cecum (15/32) and distention of the stomach with watery content (12/32). The most common microscopic finding was necrotizing enteritis (19/32), followed by mural hemorrhages in the cecum (7/32), mucosal and/or submucosal edema in large intestine (7/32) and necrotizing or heterophilic typhlocolitis (6/32). In all rabbits, typical helically coiled, gram-positive bacilli were observed on fecal or intestinal smears. *C. spiroforme* was isolated from the intestinal content of two rabbits. Failure to isolate this microorganism does not preclude a diagnosis of CSAED; *C. spiroforme* is fastidious bacterium that is difficult to isolate. Molecular testing methods are currently being developed to detect *C. spiroforme* in feces and intestinal contents of rabbits.

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84 - The multiplication of Clostridial Thioredoxin systems

Cyril Anjou[1, 2], Marie Royer[1], Aurélie Lotoux[1], Anna Zhukova[3], Léo Caulat[1, 2], Elena Capuzzo[4], Claire Morvan[1, 2], Bruno Dupuy[1], Isabelle Martin-Verstraete[1, 2]

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Thioredoxin (Trx) system is a ubiquitous protein repair machinery. In most bacteria, one or several thioredoxins reduce disulfide bonds of proteins and are then recycled by one single pleiotropic thioredoxin-reductase. However, in Clostridia, two to four complete systems (thioredoxin and reductase) are present. Our goal is to understand this atypical composition by studying the three Trx systems of *Clostridioides difficile* and their role in its lifecycle.

By performing phenotypic analysis on simple and multi-mutants, we identified that two redundant systems were involved in the resistance of the vegetative cell to infection-related stresses. However, one of them is also part of the detoxification arsenal of the spore. This spore-associated Trx system is ferredoxin-dependent, allowing activity in absence of an active metabolism, in opposition to the other systems that are classical NAD(P)H-dependent systems.

The third Trx system is part of the reductive Stickland fermentation of glycine. We showed that glycine-reductase and its associated Trx system promote sporulation, probably through consumption of glycine, a known *C. difficile* co-germinant.

Finally, we found an additional fourth thioredoxin-reductase in several *C. difficile* strains. Phylogenetic analysis demonstrated that this copy was ancestral in *C. difficile* and was lost in some clades. We showed through a trans-complementation approach that this copy is functional and involved in stress response.

Altogether, these results highlight various key roles of Trx systems in *C. difficile* physiology, and provide some clues about the multiplication of these systems by their involvement in crucial Clostridial specific mechanisms, i.e. sporulation and Stickland pathways.

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87 - *C. difficile in vitro* biofilm studies of ibezapolstat and comparator antibiotics

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Background: The high rate of *Clostridioides difficile* infection (CDI) recurrence is hypothesized to be partly attributed to biofilm formation. Ibezapolstat (IBZ) is a novel polC DNA polymerase inhibitor antibiotic with low rates of CDI recurrence in clinical trials. However, the effects of IBZ on *C. difficile* biofilms *in vitro* are unknown. The objective of this study was to test *C. difficile* anti-biofilm activity of IBZ vs. comparator antibiotics.

Methods: Antimicrobial activity (24H) and biofilm biomass (crystal violet) studies were used to evaluate four antimicrobials (IBZ, vancomycin (VAN), fidaxomicin (FDX), and metronidazole (MTZ)) on inhibition of early *C. difficile* biofilm formation (IBZ/VAN only) and *C. difficile* biofilm biomass reduction after varying biofilm formation times.

Results: Compared to control, IBZ and VAN reduced *C. difficile* growth (CFU/mL) and biomass at sub-MIC (0.4X MIC) and eradicated growth at supra-MIC (40X MIC) in early biofilms. In 24-hr biofilms, minimum bactericidal concentrations were similar to vegetative, non-biofilm growth for all four antibiotics although an Eagle effect was observed for MTZ and VAN at higher concentrations. Biomass studies for both laboratory strains demonstrated biphasic growth with reduced biomass at 48h vs. 24h and regrowth at 72h, likely due to biofilm detachment. Although differences were noted, all antibiotics in general reduced biofilm biomass compared to control regardless of biofilm growth time with highest reductions observed at later biofilm growth periods (48h and 72h).

Conclusions: IBZ was as effective as comparators to reduce biofilm-embedded *C. difficile* quantity and biofilm biomass. These results warrant the further development of IBZ.

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89 - Establishing the regulons of CmrR and CmrT, *Clostridioides difficile* response regulators involved in motility

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Clostridioides difficile introduces phenotypic heterogeneity into populations via phase variation. At eight known loci in the *C. difficile* genome, conservative-site specific recombination reversibly inverts a DNA sequence, here termed a 'switch'. The switch contains regulatory information such that its inversion modulates expression of the adjacent gene or operon in an ON/OFF manner resulting in phenotypic switching. In *C. difficile* R20291, the *cmr* switch modulates expression of the *cmrRST* operon, which encodes an atypical signal transduction system. CmrS is a putative histidine kinase, and CmrR and CmrT are response regulators with helix-turn-helix DNA binding motifs, suggesting roles in transcriptional regulation. CmrRST affects several phenotypes, including surface and swimming motility, cell chaining and elongation, biofilm formation, and virulence in the hamster model of infection. We found that CmrT is the dominant regulator for *cmr*-associated phenotypes and is required for selection of *cmr*-ON variants, whereas CmrR is primarily autoregulatory. RNA-Seq revealed that fewer than 20 genes are differentially expressed by CmrR and/or CmrT ($\log_2 > 2$, $p < 0.05$). To determine the role of these genes in CmrRST-mediated phenotypes, we constructed overexpression and deletion strains and identified two genes that are important for surface spreading and six genes that affect biofilm formation. A subset of these CmrT-regulated genes also confers selective advantages for surface growth, a condition that favors *cmr*-ON variants. Establishing the functions of these genes, and the conditions in which expression is beneficial, will provide insight on the selective pressures that influence *C. difficile* motility and virulence.

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91 - Contact-dependent inhibition of *C. difficile* sporulation by enterococci

Alicia Wood[1], Angus Johnson[1], Lesly-Hannah Gutierrez[1], Peter McKenney[1]

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Vancomycin-resistant *Enterococcus faecium* (VRE) and *Clostridioides difficile* are frequently co-isolated from human patients. We found that co-culture of VRE and *C. difficile* in liquid media results in an inhibition of the production of heat-resistant spores. Sporulation was not inhibited in conditioned VRE-conditioned medium. To determine if inhibition was contact-dependent, we developed a transwell co-culture assay and confirmed that inhibition of sporulation only occurs when VRE and *C. difficile* are in physical contact. We screened a panel of pathogenic and commensal enterococci (n=10) and found that this phenomenon is conserved with the exception of *Enterococcus saccaraolyticus*. This mode of sporulation inhibition is also conserved among *C. difficile* strains, but appears to be limited to culture in liquid media. We are currently determining the stage of sporulation inhibition and are isolating *C. difficile* mutants that are insensitive to inhibition. These data suggest that enterococci may affect the efficiency of *C. difficile* sporulation during infection.

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93 - Stickland reductive leucine metabolism links *Clostridioides difficile* metabolism with toxin production

Auriane Monestier[1, 2], Madeline Graham[3], Laura Cersosimo[3], Aidan Pavao[3], Jay Worley[3, 4], Marie Delaney[3], Lynn Bry[3], Johann Peltier[2], Bruno Dupuy[1]

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Clostridial Stickland metabolism supports rapid ATP generation and growth solely on amino acids, even in the absence of simple carbohydrates. In *Clostridioides difficile*, Stickland metabolism enables development of symptomatic infections by supporting efficient pathogen metabolism and growth. The Stickland pathways couple oxidation of donor amino acids and reduction of acceptor amino acids, such as glycine, proline, leucine. The importance of the leucine reductive pathway, encoded by the *had* operon genes, remains elusive.

We deleted the *hadA* gene in *C. difficile* strain ATCC43255, which encodes the 2-hydroxyisocaproate CoA transferase, considered the initial enzyme in the reductive leucine pathway. Short chain fatty acid measurements in the *hadA* mutant confirmed loss of the reductive leucine metabolite, isocaproate, but not the oxidative metabolite isovalerate. Absence of reductive leucine metabolism *in vitro* reduced *C. difficile*'*s* growth on amino acids in TY, in the absence of carbohydrates . However, the growth defect could be overcome with the addition of the glucose or an excess of the reductive substrate proline. In addition, deletion of *hadA* increased toxin production *in vitro* through a mechanism that remains to be identified. However, *in vivo,* germ-free mice infected with the wild-type versus Δ*hadA* strain showed 40% increased survival with the mutant per delayed early toxin production even though *in vivo* vegetative and spore biomass remained comparable with the wild-type strain. *In vivo* metatranscriptomics showed that the *hadA* mutant adapted its metabolism to sugar alcohols and mixed acid fermentations to support growth and reduced both oxidative and reductive branched chain amino acid metabolism.

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96 - The effect of tyndallized Bio-K+ probiotic bacteria on some virulence factors of *Clostridioides difficile*.

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[1]Kerry Canada

Objective: Previous work has shown that the probiotic formulation Bio-K+ (*Lactobacillus acidophilus* CL1285, *L. casei* LBC80R and *L. rhamnosus* CLR2) inhibits *C. difficile* growth, negatively impacts secretion of toxins A/B and downregulates *C. difficile* motility-related genes. The mechanism of this interaction is still unknown. In this study we are testing the hypothesis that probiotic viability is a critical parameter to achieve these effects.

Method: The probiotic preparation was tyndallized by cycles of heating/chilling immediately before using. *C. difficile* R20291 was grown in BHI with or without the tyndallized probiotic formulation for up to 24 h under anaerobic condition. Samples were taken at 0, 12 and 24 hours. CFU were enumerated on selective media. Toxins A and B were quantified by ELISA and mRNA was extracted to measure expression of *C. difficile* genes related to motility by RT-qPCR.

Results: After tyndallization of the probiotic preparation, no probiotic bacteria were enumerated. Furthermore, when cultured with the tyndallized probiotic preparation, no change in growth of *C. difficile* or toxin production was observed. Swimming assays were assessed and no changes in the motility of *C. difficile* were observed. Finally, the genes related to the motility were not modulated by the presence of the tyndallized probiotic formulation.

Conclusion: These results confirm our hypothesis that viability of the Bio-K+ probiotic formulation is necessary to impair critical virulence factors used by *C. difficile* to infect a host.

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98 - The role of extracellular polymers in *C. difficile* mucosal adhesion

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Mucins are glycoproteins which can be found in host cell membranes and secreted to form mucus, a gelatinous hydrogel. Mucosal surfaces in mammals form a barrier to invasive microbes, particularly bacteria, but are a point of attachment for others. While *Clostridioides difficile*, a bacterium which colonizes the mammalian GI tract, leading to a variety of negative outcomes, is known to associate with the mucus layer and underlying epithelium, the mechanisms underlying these interactions that facilitate colonization are less well-understood. To understand the molecular mechanisms by which *C. difficile* interacts with mucins, we used *ex vivo* mucosal surfaces to test the ability of *C. difficile* to bind to mucins from different mammalian tissues. We found significant differences in *C. difficile* adhesion based upon the source of mucins, and this binding was dependent upon mucin glycosylation as both chemical removal of O-linked glycans and pre-incubation with mucus-degrading commensal microbes decreased binding by *C. difficile*. We also observed defects in adhesion by a *C. difficile* R20291 fliC (flagellin) mutant but not in a pilA1 mutant (the major subunit of type IV pili). Robust adhesion was observed for a variety of strains, including a non-motile clade 5 strain with no correlation observed between adhesion to mucus and swimming motility. These results imply that *C. difficile* flagellin facilitates the initial host attachment of *C. difficile* to secreted mucus, and potentially to membrane-bound mucins on host cells. But it remains to be determined whether this interaction is direct or mediated by the production of other adhesins.

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100 - Genetic engineering of *Clostridioides difficile* bacteriophages

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Clostridioides difficile (*Cd)* is a major cause of healthcare-associated diarrhea in industrialized countries. The standard treatment for *Cd* infections is antibiotherapy, which causes dysbiosis that promotes infection recurrence. As such, there is an urgent need for more targeted treatments to cure *Cd* infections. A potential candidate is the use of lytic bacteriophages (or phages) that lyse the pathogen in a very specific way while being harmless to the indigenous gut residents. However, *Cd* phages have two main hurdles limiting their potential therapeutic use, i.e. their narrow host spectrum and the fact that all of them are temperate, meaning that they can integrate their DNA within their host's genome during the lysogenic cycle, sparing it and protecting it from subsequent infection by similar phages. This project aims to solve these issues by genetic engineering of *Cd* phages. First, we identified the *receptor binding protein* (RBP) and key lysogenic genes, like the *cI* repressor and the integrase, in phage genomes using bioinformatics and/or functional overexpression assays. Using an allelic exchange method exploiting *Cd's* endogenous CRISPR-cas system, we successfully exchanged two phage RBP genes creating a recombinant phage with a modified host spectrum. Using a similar approach, we are working on the deletion of the *cI repressor* and *integrase* genes from phage genomes to prevent the use of the lysogenic cycle, thus creating strictly lytic phages. This project will lead to a better understanding of phage-host interaction and will eventually lead to the creation of therapeutic phages to treat *Clostridioides difficile* infection.

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102 - Evaluation of AlphaFold-Multimer for the prediction of the *Clostridioides difficile* PolC-type DNA polymerase III alpha- and beta-subunit protein-protein binding

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Background: AlphaFold-Multimer (AFM) is a recently developed deep learning algorithm that predicts protein tertiary and quaternary structures. The purpose of this project was to assess AFM to predict the 3D protein quaternary structure of the *Clostridioides difficile* PolC-type DNA Polymerase III α- and β-subunit complex (CdiPolC/β).

Methods: The *C. difficile* R20291 CdiPolC/β primary sequences was obtained using CLC Genomics (Qiagen) and modeled (1:2 stoichiometry) using AFM on an MMseqs2 ColabFold notebook (Template mode:none; MSA mode:mmseqs_uniref_env; pair mode:unpaired_paired). The resultant structure was compared to that of *E. coli* (PDB 5FKV) using the Dali Server.

Results: AFM predicted with high accuracy (pLDDT>90) the quaternary structure of the CdiPolC/β, except for the 3'-to-5' exonuclease domain (pLDDT<50) due to removal from prior structures. Upon visual inspection, CdiPolC had the classical right-handedness of C-family DNA polymerases and comparable overlap (3.4 Å RMSD) to *E. coli* Pol IIIα despite limited sequence identity (24%). The *C. difficile* β-clamp formed the correct head-to-tail dimeric ring with domain I bound to domain III of the opposite monomer similar to that of *E. coli* (1.7 Å RMSD) with limited identity (27%). The PolC and β-clamp interact through the PolC β-binding motif (^{1428}QLSLF^{1432}), similar to that of *E. coli* Pol IIIα (^{920}QLDLF^{924}).

Conclusion: AlphaFold-Multimer (AFM) was able to predict the quaternary structure of the *C. difficile* PolC α-subunit and β-clamp bound together despite the absence of structural data from this species. Further work to validate this structure will aid the development of novel antibiotics that target the *C. difficile* PolC.

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103 - Molecular Mechanisms Underlying Mucosal Attachment and Colonization by *Clostridioides difficile*

Armando Lerma[1], Benjamin Sidner[1], Jennifer Auchtung[1], Kurt Piepenbrink[1]

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Clostridioides difficile has become one of the leading causes of nosocomial gastrointestinal infections and an urgent threat to public health. Infection typically occurs after antibiotic treatments, however, infections which are not preceded by antibiotic use have become increasingly more common in community-associated settings. While this pathogen has been widely studied, the molecular mechanisms by which C. difficile is able to colonize and persist in the gut are still not completely understood. Mounting evidence has suggested that C. difficile interacts with the outer mucus layer during infection and might be essential for colonization, therefore we used an in vitro mucus layer model to help understand C. difficile's binding and colonization mechanisms to the host. We used gene-interruption mutants of the major subunits of two extracellular appendages that have been implicated in adhesion, type IV pili and flagella, to demonstrate that the presence of flagella facilitates initial attachment to the mucus layer. We also observed that the attachment varied across multiple strains of C. difficile and was dependent on the source of mucin derivation. Attachment was decreased when mucin-degrading bacteria modified existing glycans in our model. These results suggest that adherence to mucin potentially determines C. difficile's ability to colonize and may open the door to new strategies to combat C. difficile infection.

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104 - Visualizing virulence and transmission gene expression in *Clostridioides difficile*

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Clostridioides difficile is an anaerobic, spore-forming, bacterial pathogen that is the most common cause of healthcare-associated infections in the United States. *C. difficile* is transmitted by spores through the fecal-oral route, and upon reaching the colon the spores germinate into metabolically active vegetative cells that produce toxins and damage the intestinal epithelium. Despite toxins being essential for causing damage to the host, toxin levels and *C. difficile* biomass measured in feces during murine infection do not correlate with disease severity. These findings indicate how our understanding of factors determining disease severity during *C. difficile* infection (CDI) is limited. Previous work indicates that *C. difficile* exists in two subpopulations during murine infection, with most being found in the gut lumen and a subset being located near the colonic epithelium. Recent analyses suggest that this epithelium-proximal sub-population may modulate disease severity and thus explain the lack of correlation between toxin levels and biomass in feces and clinical outcomes. With the role of epithelium-associated populations of *C. difficile* during CDI being unclear, my work is focused on testing the hypothesis that the spatial distribution of *C. difficile* within the colon is a key factor in determining disease severity. To address this hypothesis, I have constructed fluorescent reporter strains to visualize the spatial distribution of *C. difficile* as well as toxin gene expression during murine infection. Additionally, I will make deletion mutants of candidate genes implicated in driving association with the gut epithelium to determine genetic factors in *C. difficile* that determine disease severity.

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106 - Unexpected strain and toxin diversity of soil *Clostridioides* in pristine environments in Costa Rica

María Paula Bolaños[1], Eliott Bosshard[2], Daniela Aguilar[1, 3], César Rodríguez[1, 3]

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Clostridioides difficile gained notoriety due to the dissemination of strains catalyzed by human practices. However, its epidemiology is constantly changing due to its vast genetic plasticity and associations with animals and food, heightening the risk of community exposure.

While sporadic evidence of *C. difficile* and other *Clostridioides* species in soil exists, their lack of toxin genes often leads to their neglect as a potential health threat. This study explores this underexamined niche, aiming to enhance our understanding of the evolutionary trajectories of these organisms.

We collected soil from 15 undisturbed sites in Costa Rica and cultured *C. difficile* following a spore enrichment procedure. The isolates were characterized using latex agglutination, MALDI-ToF, API, GDH, and toxin B detection tests, and whole-genome sequencing. We recovered isolates from three *Clostridioides* species that we previously detected in Costa Rican hospitals, with almost half of them harboring alleles of toxin B (TcdB) or binary toxin that displayed < 90% amino acid identity with canonical alleles. These toxin genes were located within diverse genetic contexts, including conjugative plasmids and prophages that could facilitate their lateral transfer. Most isolates corresponded to novel sequence types (STs), with several STs identified at a single site, indicating a high level of diversity within these species.

Our findings underscore that soil serves as a recombination platform for a myriad of toxigenic *Clostridioides* species. This notion challenges paradigms on the epidemiology and ecology of TcdB-associated pathologies and reaffirms the need for a One Health approach in their study.

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108 - Stopping a killer superbug: unravelling the molecular mechanisms of *C. difficile* sporulation

Charlotte G. Roughton[1], Diogo Martins[2], Daniela Vollmer[1, 3], Joe Gray[1], Anna Barwinska-Sendra[1], Waldemar Vollmer[1, 3], Adriano O. Henriques[2], Monica Serrano[2], Paula S. Salgado[1, 3]

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Clostridioides difficile is an antibiotic resistant bacterial human pathogen that colonises the large intestine in the absence of a healthy competing gut microbiota. Current treatments for *C. difficile* infection (CDI), the most common cause of healthcare-associated gastrointestinal disease, entail use of antibiotics that perpetuate gut dysbiosis and enable disease recurrence. This highlights an urgent requirement to further understand the fundamental biology of this pathogen so novel therapies can be sought.

A promising, albeit unexploited, target for new therapeutics is sporulation, which begins with an asymmetrical cell division, and entails substantial peptidoglycan remodelling as the smaller forespore is engulfed by the larger mother cell prior to spore maturation.

SpoIIP is a dual amidase and endopeptidase involved in peptidoglycan remodelling during engulfment. Previous work has shown that SpoIIP is present in more than one form, either a full-length isoform associated with the membrane or shorter forms detected in soluble fractions. Here, we generated and biophysically characterised different variants and tested their enzymatic activity using peptidoglycan digestion assays. Our data shows that a shorter isoform, corresponding to residues 168-399, is inactive whilst a 98-339 intermediate form, retains amidase and endopeptidase activity. Moreover, the shorter form also exhibits lower stability and a less ordered structure.

This work contributes to the understanding of the molecular details of sporulation to find potential therapeutic targets to interrupt spore formation, thereby eliminating the CDI transmission route.

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109 - Identifying the cellular receptor for *Clostridium perfringens* NetF

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Clostridium perfringens causes severe diseases in animals and humans. It produces a large arsenal of virulence factors, many of them belonging to the hemolysin-like family of β-pore forming toxins (β-PFTs). Knowledge about role and action of many of these toxins is still limited. Our discoveries of CD31 as the membrane receptor for *C. perfringens* beta-toxin (CPB) and our elucidation of CPB pore structure using cryo-EM have made important contributions to the knowledge on this family of proteins. Recently, we extended our research to other clostridial hemolysin-like β-PFTs and generated preliminary data on the receptor and cell-type specificity of Necrotizing Enteritis Toxin F (NetF). NetF has 32.64% sequence identity and 51.34% sequence similarity with CPB and a predicted molecular weight of 34 kDa. NetF was first described in 2015 and associated with *C. perfringens* type A strains causing hemorrhagic enteritis in dogs and foals. Using a comparative RNAseq approach on susceptible and resistant cells, we identified Capillary Morphogenesis Protein 2 (CMG2 or ANTXR2) to be important in NetF-mediated cytotoxicity. CMG2 is also one of the two known receptors for protective antigen (PA) of *Bacillus anthracis* anthrax toxin. We demonstrated that CMG2 expression on target cells is essential for NetF toxicity and that PA competitively inhibits NetF cytotoxicity. To further investigate the interaction of NetF with its putative membrane protein receptor, we engineered mutant versions of CMG2 lacking parts of its extracellular domain. Our results may highlight molecular mechanisms and structures that confer receptor-, cell-type and species specificity for clostridial hemolysin-like βPFTs.

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110 - L,D-transpeptidases and peptidoglycan cross-linking in *Clostridioides difficile*

Ana Oliveira Paiva[1], Pascal Courtin[2], Olga Soutourina[1], Marie-Pierre Chapot-Chartier[2], Johann Peltier[1]

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Although resistance of *C. difficile* to β-lactams is a leading contributor to the development of *C. difficile* infection, the underlying mechanisms of resistance are still largely unknown. Thus, the contribution of β-lactamases to β-lactam resistance was recently shown to be very modest in *C. difficile*. *C. difficile* has an original peptidoglycan (PG) structure with mainly 3-3 cross-links to the detriment of the widespread 4-3 cross-links. The 3-3 cross-links are synthesized by non-classical transpeptidases, namely the L,D-transpeptidases (LDTs). All LDTs identified so far share the conserved YkuD catalytic domain and are not efficiently inhibited by most β-lactams. This suggests that LDTs could contribute to β-lactams resistance in *C. difficile*. *C. difficile* encodes three putative LDT, Ldt1, Ldt2 and Ldt3, with the YkuD domain. In this study, we generated a combination of single, double and triple deletion mutants of the corresponding genes and the triple mutant was confirmed via whole genome sequencing. Vegetative cells PG was purified from the different *C. difficile* strains grown in TY, digested with mutanolysin and the muropeptides analyzed by RP-UHPLC. Surprisingly, 3-3 cross-link formation was not abolished in vegetative cells of the triple mutant and only mutation of *ldt1* affected their abundance. Structure of the spore PG was also determined. The spore PG is loosely cross-linked but 3-3 cross-links could still be identified. However, spore PG cross-linking remained unchanged in the triple mutant. These results suggest the presence of at least a fourth LDT containing a novel catalytic domain. Work is underway to tentatively identify this enzyme.

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112 - Prior host exposure to matched microbiota improves success of fecal microbiota transplantation for clearing *Clostridioides difficile* in mice

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Successful treatment of *Clostridioides difficile* infection (CDI) using fecal microbiota transplantation (FMT) is associated with restoration of a diverse gut microbiota. Although it is typically assumed that restoring microbiota composition equates to functional restoration of colonization resistance against *C. difficile*, our previous data in a model of recurrent CDI suggests that compositional recovery alone is not sufficient for clearance. This was demonstrated by an inability of healthy human feces to clear *C. difficile* in specific-pathogen-free mice, despite its ability to restore diverse microbes. Metagenomic analysis additionally demonstrated recovery of gene-encoded functions typically associated with *C. difficile* clearance. To assess if prior exposure to specific microbiota was necessary to “prime” mice for successful FMT-based clearance of *C. difficile*, we conducted our recurrence model in mice with humanized microbiota. Germ-free mice initially inoculated with different human feces (n=6) demonstrated variable resistance to direct *C. difficile* challenge, with increased abundance of *Lachnospiraceae* and *Ruminococcaceae* species correlating to *C. difficile* resistance. Importantly, ex-germ-free mice colonized with a resistant human microbiota were now amenable to *C. difficile* clearance during recurrent CDI when treated with FMT matching their baseline feces. This same resistant fecal community was still unable to clear *C. difficile* in SPF mice, suggesting that initial exposure or adaptation with a particular microbiota may influence FMT outcome. Future studies will focus on identifying functional differences between these two conditions, related to both the microbial and immune status.

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114 - Transition to a lean *C. difficile* sentinel surveillance program in the Netherlands and a persisting trend of more severe disease

Joffrey van Prehn[1, 2], Wiep Smits[1, 2], Karuna Vendrik[2, 3], Shady Gaber[1], Daan Notermans[2, 3], Ed Kuijper[1, 2], National CDI Working group[1, 3, 4, 5, 6, 7]

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Clostridioides difficile ribotype (RT)027 outbreaks from 2005 onwards led to a Dutch sentinel surveillance program in 2009 by the National Reference Laboratory (NRL) to monitor the incidence of *C. difficile* infections (CDI) in hospitalized patients. Since 2022, the NRL continued as Expert Center for CDI.

Sentinel surveillance was continued in three academic and two secondary care centers geographically spread throughout the Netherlands (previously 22-24 centers). *Ad hoc* typing for severe CDI cases or suspected outbreaks continued to be offered for all laboratories. Here we present data from the last 4 years.

CDI incidence remained stable with 3.1/10,000 patient days (95%CI 2.8-3.4) in 2018-2019 (pre-COVID19) compared to 3.2/10,000 (2.9-3.5, n=290 cases) in 2022. CDI-attributable mortality was also stable: 1.1% (0.4-1.8) in 2018-2019 and 1.0% (0.0-2.2) in 2022. The proportion of patients with severe disease increased from 16.1% (13.7-18.5) in 2018-2019 to 30.3% (25.0-35.6) in 2022. Limiting the 2018-2019 analysis to the five currently participating centers, the proportion severe CDI increased from 15.0% (11.1-18.9) to 30.3%. RT027 prevalence was 0.8% (0.6% in 2018-2019). RT014/020 continued to be the most prevalent: 16.7% in (19.5% in 2018-2019). We received 42 samples for *ad hoc* typing. No outbreaks were reported in 2022

The trend of more severe CDI cases reported in the Netherlands at the end of 2021 (PMID35782989) continued. Ribotype distribution and CDI incidence with the new sentinel program mirrored previous (pre-COVID-19 and COVID-19) years. Currently, further epidemiological analysis into the increase of severe CDI is conducted and cgMLST typing is being implemented.

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120 - Probing the *Clostridioides difficile*-host interface during infection

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Clostridioides difficile is a significant cause of hospital-acquired gastrointestinal infections. The recent rise of community-acquired *C. difficile* has increased the burden on global healthcare services due to its inherent antibiotic resistance coupled with a propensity to cause recurrent infections. *C. difficile* colonisation of the gut is an essential determinant of bacterial carriage and disease outcome; however, we lack knowledge about bacterial or host factors modulating interactions between *C. difficile* and the gut epithelium.

To understand these interactions occurring at the interface, we recently performed a dual RNA-seq analysis of *C. difficile* infection in an in vitro human gut model, which revealed changes in the expression of several bacterial and host genes. Cell wall protein 10 (Cwp10) was one of the most highly induced *C. difficile* genes during early infection. To study this previously uncharacterised protein, we created isogenic mutants in *C. difficile* R20291. The *cwp10* mutant interestingly demonstrates decreased adhesion to gut epithelial cells as determined by colony counts and microscopy from multiple in vitro cell infection assays; whilst displaying increased biofilm formation. The lack of *cwp10* did not impact cell hydrolysis or toxin production but was associated with mild defects in spore germination. Adhesion assays with immobilised extracellular matrix (ECM) components indicate that Cwp10 is involved in binding to specific host ECM proteins. We are currently investigating the role of Cwp10 in *C. difficile* colonisation in murine infection models.

Our data indicate that Cwp10 is a new adhesin that facilitates C. difficile attachment to the gut epithelium during infection.

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122 - Expanding upon a network of sRNAs regulating sporulation in *C. difficile*

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The ability to form endospores allows *Clostridioides difficile* to resist antibiotic treatment and to persist in the intestinal tract. Sporulation initiation has to be tightly controlled because it ultimately leads to the death of the mother cell. However, the exact mechanism or environmental stimuli that regulate the activity of the master transcriptional regulator Spo0A are incompletely understood.

Using RIL-seq (RNA interaction by ligation and sequencing), our group recently established that the sRNAs SpoY and SpoX inversely regulate sporulation by modulating Spo0A production through base-pairing to *spo0A* mRNA (Fuchs et al., 2023). Moreover, the RIL-seq dataset predicted additional interactions between the *spo0A* mRNA and 9 other sRNAs suggesting a complex post-transcriptional network of RNA regulators that modulate sporulation initiation. We detected distinct expression patterns of these sRNAs in different growth conditions hinting at a potential mechanism by which environmental signals are translated into a cellular response. Interestingly, three of these sRNAs have in turn been identified as interaction partners of SpoX. It is likely that either SpoX or the remaining sRNAs sequester each other to fine-tune translation and thus sporulation initiation in *C. difficile*.

Therefore, we will study factors that impact expression, function and stability of these sRNAs, including potential sponging activities. Furthermore, since sporulation is an asynchronous cellular process, we will explore whether ncRNAs regulate the same targets across cells and how heterogeneity contributes to regulatory outcomes using single cell approaches.

Characterizing the regulatory mechanisms of this sRNA network represents a promising approach for gaining key insights into sporulation initiation.

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124 - Development and validation of LC-MS/MS method to determine omadacycline concentrations in human fecal samples

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Background: Omadacycline has potent in vitro activity against *Clostridioides difficile* however fecal pharmacokinetics of omadacycline in humans is not well-described. This study aimed to develop and validate a liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based methods to determine fecal concentrations of omadacycline.

Method: Chromatographic separation of omadacycline was performed on a BEH C18 column by gradient elution using a mobile phase A (methanol) and a phase B (ammonium formate and formic acid). Quantification was carried out with transitions of 557.300-453.100 (omadacycline) and 566.400-453.100 (internal standard). Standard curve was created using a series of omadacycline solutions in fecal matrix with intra- and inter-day accuracy and recovery evaluated. Clinical fecal samples from an ongoing phase I study were treated with methanol, water, and EDTA. Extracted omdacycline was diluted by 100-400 fold prior to analysis.

Results: The omadacycline peak was separated within 5 minutes with a low limitation of detection (0.03 ng/ml) and the limitation of quantification (0.1 ng/ml). The intra- and inter-day accuracy and recovery were >90%. The fecal concentrations of omadacycline in the stool samples (n=93) averaged 33.9±43.9 µg/ml (range: undetectable-195.2 µg/ml). A significantly higher proportion of 4-epimer omadacycline was observed in clinical trial fecal samples compared to spiked samples.

Conclusion: We developed a LC-MS/MS-based method to determine fecal concentrations of omadacycline and validated using the clinical fecal samples. This assay may be considered in future clinical trials and, further studies may be warranted to better understand the significance of the 4-epimer omadacycline.

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126 - Fecal Microbiota Transplantation Stimulates Acute Immune Activation but Promotes Type 2 Immune Responses in an Antibiotic Mouse Model

Brett Moreau[1], Farha Naz[1], William A. Petri, Jr.[1]

[1]University of Virginia

Clostridioides difficile infection (CDI) is the leading hospital acquired infection in North America. Previous studies have identified Type 2 immune responses as critical for reducing tissue damage and mortality during CDI. While previous work on fecal microbiota transplantation (FMT), a highly effective treatment for CDI, has focused on colonization resistance mounted against *C. difficile* by FMT-delivered commensals, the effects of FMT on host gene expression are poorly understood. To address this gap in knowledge, gene expression was assessed after FMT in an antibiotic mouse model. FMT administration led to a significant increase in alpha ($p<0.001$) and beta ($p=0.001$) diversity and partial restoration of microbiome structure within 48 hours. Bulk RNA sequencing of cecal tissue at this early timepoint identified 864 differentially expressed genes between FMT-treated and control mice. Strikingly, the FMT-treated group was enriched in immune pathways, particularly pro-inflammatory responses. Simultaneously, several Type 2 immune genes were upregulated, including *IL33* (LogFC=1.6, $p<0.001$), *SOCS3* (LogFC=1.8, $p<0.001$), and *IL1RL1* (LogFC=0.9, $p=0.02$). To evaluate whether these changes persist at later timepoints, immunoprofiling was performed using flow cytometry at seven days post-FMT. While CD45+ immune cell counts were significantly elevated ($p=0.015$) in the colon of FMT-treated mice, these cells exhibited dampened Type 1 and heightened Type 2 responses, with lower percent abundance of Ly6c-high monocytes and neutrophils and higher percent abundance of eosinophils and alternatively activated macrophages. These results highlight the impact of FMT on host gene expression, providing evidence that FMT can facilitate Type 2 immune responses, which are ultimately beneficial during CDI.

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146 - *Clostridioides difficile* Infection Promotes Gastrointestinal Dysfunction in Human and Mice Post-Acute Phase of the Disease

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In the US, *Clostridioides difficile* (*C. difficile*) infection (CDI) is the 8th leading cause for hospital readmission and 7th for mortality among all gastrointestinal (GI) disorders. Here, we investigated GI dysfunction post-CDI in human and mice post-acute infection. From March/2020 to July/2021, 67 patients were referred to the UVA Complicated *C. difficile* Clinic and clinical records were analyzed. In vivo, C57BL/6 mice were infected with *C. difficile* (VPI10463) and clinical score were determined daily. Stool samples were collected to analyze the shedding of *C. difficile* and myeloperoxidase (MPO) levels. On day 21 post-infection, Evans's blue and FITC-70kDa method were performed to evaluate GI motility. Of the 67 patients, 40 patients (59.7%) were diagnosed with CDI, and 22 patients (32.8%) with post-CDI IBS (diarrhea-type, constipation-type, and mixed-type). In infected mice, levels of MPO on stools and clinical score were higher on day 3. On day 21, mice recovered from body weight loss induced by CDI, and no detectable MPO was found. The total GI transit time (TGITT) and FITC-70kDa levels on proximal colon increased on infected mice (p=0.002), suggesting a constipation phenotype post-acute phase of CDI. A positive correlation on intestinal inflammation on day 3 and TGITT on day 21 was observed. In conclusion, post-infection intestinal dysfunction occurs in human and mice post-CDI. Importantly, we have validated in the mouse model that CDI causes abnormal GI transit in the recovery phase of the disease, indicating the potential utility of the model in exploring the underlying mechanisms of post-infectious IBS in humans.

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147 - Pathology and molecular diagnosis of *Clostridium colinum* infection in Quail from California (1992-2022)

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Clostridium colinum causes ulcerative enteritis in several avian species. The disease is particularly prevalent in quail, and it is therefore colloquially known as “quail disease”. The pathogenesis of the infection is poorly understood. A retrospective study of 27 cases of *C. colinum* infection in quail, submitted for necropsy and diagnostic work up to the California Animal Health and Food Safety Laboratory System between 1992 and 2022 was performed. The necropsy reports were reviewed and PCR of *C. colinum* (16S rRNA) was performed on formalin-fixed, paraffin embedded tissues. Grossly, the following lesions were observed: intestinal ulceration (27/27, 100%) affecting duodenum (25/27, 93%), jejunum (2/27, 7%) and/or cecum (5/27, 19%), muscle atrophy (23/27, 85%), and hepatic necrosis (9/27, 33%). Histologically, 27/27 (100%) quails showed multifocal ulcerative enteritis, typhlitis and/or colitis, with intralesional bacilli, which in 4/27 (15%) of the cases was associated with celomitis, and hepatic necrosis (7/27, 33%). *C. colinum* was detected by PCR in all 27 (100%) cases, but isolated from the intestine of only three (11%) cases. These results suggest that diagnosis of *C. colinum* infection should be performed based on gross and microscopic lesions, coupled with PCR. Culture has very low sensitivity for the identification of this microorganism, which is consequence of the fastidious nature of *C. colinum*.

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149 - Novel insights in the biosynthesis of the *Clostridioides difficile* 630Δ*erm* flagellin C (FliC) Type A modification

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Most bacteria are flagellated, i.e. they have at least one flagellum. Rotation of the filament of flagella allows directed motility towards beneficial conditions while leaving non-favorable environments. Moreover, flagella mediate processes like adherence and immunomodulation. In the *C. difficile* strain 630Δ*erm*, the main filamentous component, FliC, is post translationally modified with an *O*-linked Type A glycan structure. The modification of FliC with the Type A glycan is essential for flagellar function since motility is seriously impaired in individual gene mutants with improper biosynthesis of the flagellar glycan. A cluster of genes (*cd0240-cd0244*) has been assigned to be involved in the biosynthesis of the Type A structure, but the role of each individual gene, and the corresponding enzymatic activity, has so far not been fully elucidated. By performing new quantitative mass spectrometry-based proteomics analyses, we determined the relative abundance of the observed variations of Type A structure in the individual gene mutants. For the first time, we show that one of the involved proteins, CD0244, is essential for the biosynthesis of the Type A modification. Together with additional bio-informatic analyses, we predict the functions and enzymatic activities of CD0244 and of other proteins involved in Type A biosynthesis, resulting in a new model for the Type A glycan biosynthesis.

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151 - Exploring botulinum neurotoxin export in *Clostridium botulinum* Hall A

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Clostridium botulinum is known for its production of an extremely potent neurotoxin that is the causative agent of the potentially fatal illness botulism. Botulinum neurotoxin (BoNT) is recognised as the most toxic natural substance known to humankind and is exploited for numerous therapeutic and cosmetic applications. Despite its great importance to public health, pharmacology and bioterrorism threat, the means by which the BoNT is released from the bacterial cell has not been fully elucidated.

Historically the presence of the toxin in the extracellular media was considered a consequence of cell lysis. However, evidence points towards the export of the toxin. The possible mechanisms for toxin export were explored through precise gene deletions in the model Group I strain of *C. botulinum* Hall A, ATCC 3502. Genes encoding flagellar structural and regulatory components, pilus components and holins were individually deleted, and mutants were phenotypically characterised. The concentration of extracellular BoNT was analysed and significantly reduced in flagella mutants, whereas extracellular concentrations remained comparable to the wildtype and in the other mutant strains created. This work provides evidence to establish a link between the flagella and the regulation of the BoNT in *C. botulinum.*

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