The co-directors of this project are:
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Among gastrointestinal infections, which represent a major public health issue in both developed and developing countries, the Gram-positive, anaerobic, spore-forming bacterium *Clostridioides difficile* is the leading cause of intestinal healthcare-associated infections and life-threatening fulminant colitis in adults receiving antibiotic treatments in Europe and the U.S. The Centers for Disease Control and Prevention (CDC) considers *C. difficile* one of the top three urgent antibiotic resistance threats. Strains of *C. difficile* that are less susceptible and/or resistant to the typical antibiotic treatments have recently emerged (1), highlighting the need for new therapeutic strategies.
Bile acids (BAs) (cholic acid (CA) and chenodeoxycholic acid (CDCA)) are small molecules synthesized from cholesterol by the liver. They are then conjugated with taurine or glycine to form conjugated BAs, which are excreted to facilitate lipid solubilization and absorption. There is a close relationship between BAs and intestinal microbes. Some bacteria can deconjugate BAs, releasing the amino acid and the deconjugated BAs (2). BAs can also be converted from primary to secondary BAs (deoxycholic acid (DCA) or lithocholic acid (LCA)) in the colon by bacteria encoding 7α/β-dehydroxylation enzymes (3) or by isomerization of the hydroxyl (-OH) group in position 7 or 3 by bacteria encoding β-hydroxysteroid dehydrogenase (4).
The equilibrium between different BAs provides either a favorable or an unfavorable environment for pathogens like *C. difficile*. Primary BAs, such as taurocholate, are known to promote *C. difficile* spore germination into vegetative cells, while secondary BAs (deoxycholate and lithocholate) generally inhibit germination and *C. difficile* growth (5). Lithocholate and its isomers alter different aspects of the *C. difficile* life cycle, including growth, toxin expression, and activity, while maintaining host cell integrity and sparing the intestinal microbiota (6). Additionally, Partner #2 has shown that some commensal species of *Clostridia*, through differential enrichment of primary BAs and metabolites, can reduce or exacerbate *C. difficile* virulence in murine models (7). These modified BAs can have either pro- or anti-inflammatory properties with prolonged impacts on microbiota function. Partner #1 has previously reported that in inflammatory bowel disease (IBD) patients, there is an increase in conjugated primary BAs in feces and a decrease in secondary BAs in serum and feces (8). They also observed a defect in BA isomerization in IBD patients, suggesting a strong link between BA isomerization levels and inflammation. Furthermore, ursodeoxycholic acid (UDCA), a secondary modified BA of interest, is a bioproduct of bacterial isomerization. UDCA exhibits anti-inflammatory properties by lowering IL-8 secretion in human epithelial cells exposed to pathogenic *E. coli* strains (Partner #1).
Our understanding of the role of BAs and their metabolism in *C. difficile* infection and inflammation is still incomplete. Thus, we propose to pursue this study with targeted approaches involving various bile acids (Partner #1), the isobiotic OMM12 mice (a controlled murine model), and the opportunistic intestinal pathogen *C. difficile* (Partner #2). This model was chosen because it harbors and transmits a stable population of 12 bacterial species in axenic (C57BL/6) mice. Importantly, Partner #1 and #2 have shown that in OMM12 mice, primary BAs exceed secondary ones (mean ratio: 2.53 ± 2.30) since, after deconjugation, primary BAs cannot be further modified due to the absence of 7α/β-dehydroxylation enzyme activity in the genomes of the 12 resident bacteria. Therefore, OMM12 mice, being amenable to strain addition, provide a platform for testing the contribution of specific microbes encoding such enzymes to BA metabolism. Partner #2 has already demonstrated that *C. difficile* successfully colonizes the gastrointestinal tract (GIT) of OMM12 mice for at least 20 days, while in specific pathogen-free (SPF) mice, *C. difficile* levels decline sharply within seven days post-infection.
We will enrich the microbiota function of the OMM12 mice by introducing bacteria that complete BA metabolism. Partner #1 possesses bacterial strains in its collection with demonstrated in vitro abilities to transform bile salts. This model overcomes the technical barrier of working with conventional mice, in which multiple bacteria can redundantly perform BA modifications. The ability to produce specific BAs in vivo at relevant concentrations will allow us to determine their respective impact on bacterial community structure and host response.

We propose an initial in vitro approach to BA-driven modulation of inflammation. We will expose cellular models to secondary and/or isomerized BAs to evaluate their direct pro-inflammatory impact (Partner #1). The same assay will be performed under a pro-inflammatory environment to identify BAs with anti-inflammatory properties. The pro-inflammatory environment will be induced by whole extracts of different *C. difficile* strains producing low or high levels of toxins known to trigger inflammation (Partner #1 and #2).

Next, to validate the influence of BAs on the OMM12 ecosystem composition and *C. difficile* infection, the 12 OMM12 strains will be grown individually and mixed to reconstitute the bacterial consortium in vitro. Primary, secondary, or isomerized bile salts will be added to assess their effects on the overall bacterial composition. Then, strains producing secondary or isomerized bile salts will be introduced to the consortium alongside a primary bile salt. The impact of these additions on bacterial ecosystem composition and bile salt profiles will be determined (Partner #1). *C. difficile*strains will be further introduced into this in vitro model to determine the effect of bile salt metabolism on *C. difficile*(Partner #1 and #2).

Finally, OMM12 mice will be colonized with key bacterial players in the sequential metabolism of BAs to convert primary BAs into dehydroxylated and isomerized secondary BAs (Partner #2):

1. One species that performs 7-dehydroxylation will be introduced into OMM12 mice, yielding the OMM13Em model.
2. One species will then be added to OMM13Em mice to achieve isomerization at position 3α, resulting in the OMM14 model.
3. Independently, OMM12 mice will be colonized with one species that performs 7α-isomerization, creating the OMM13Cs model.

The success of gut implantation and colonization by these strains will be monitored using qPCR. The BA pool in fecal and serum samples from OMM12, OMM13Em, OMM13Cs, and OMM14 mice will be analyzed by HPLC-MS/MS to assess qualitative and quantitative profiles (Partner #1 and #2). *C. difficile* will then be introduced into OMM13Em, OMM13Cs, and OMM14 mice. BA profiles will be analyzed, and in all OMM models, markers of inflammation and bacterial composition will be measured (Partner #1 and #2). The impact of intestinal BA metabolism on *C. difficile* infection will be evaluated by comparing healthy and *C. difficile*-infected mice (Partner #1 and #2).

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