

Inhibition of spore germination, growth, and toxin activity of clinically relevant *C. difficile* strains by gut microbiota derived secondary bile acids



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ABSTRACT

The changing epidemiology of *Clostridium difficile* infection over the past decades presents a significant challenge in the management of *C. difficile* associated diseases. The gastrointestinal tract microbiota provides colonization resistance against *C. difficile*, and growing evidence suggests that gut microbial derived secondary bile acids (SBAs) play a role. We hypothesized that the *C. difficile* life cycle; spore germination and outgrowth, growth, and toxin production, of strains that vary by age and ribotype will differ in their sensitivity to SBAs. *C. difficile* strains R20291 and CD196 (ribotype 027), M68 and CF5 (017), 630 (012), B19 (001) and M120 (078) were used to define taurocholate (TCA) mediated spore germination and outgrowth, growth, and toxin activity in the absence and presence of gut microbial derived SBAs (deoxycholate, isodeoxycholate, lithocholate, isolithocholate, ursodeoxycholate, ω -muricholate, and hyodeoxycholate) found in the human and mouse large intestine. *C. difficile* strains varied in their rates of germination, growth kinetics, and toxin activity without the addition of SBAs. *C. difficile* M120, a highly divergent strain, had robust germination, growth, but significantly lower toxin activity compared to other strains. Many SBAs were able to inhibit TCA mediated spore germination and outgrowth, growth, and toxin activity in a dose dependent manner, but the level of inhibition and resistance varied across all strains and ribotypes. This study illustrates how clinically relevant *C. difficile* strains can have different responses when exposed to SBAs present in the gastrointestinal tract.

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1. Introduction

Clostridium difficile is a Gram-positive, spore forming, anaerobic bacillus and is the leading cause of nosocomial infection worldwide [1]. In the United States half a million cases of *C. difficile* infection (CDI) and 29,000 deaths are reported annually [2,3]. Clinical disease can range from mild or moderate diarrhea to fulminant and pseudomembranous colitis and even death [4,5]. Incidence, severity, mortality, and recurrence rates of CDI have increased during the past 15 years. The changing global epidemiology of CDI has been largely attributed to the emergence of epidemic *C. difficile* strains, PCR ribotype 027 and 078. Other PCR ribotypes such as 001, 053,

and 106 have also been associated with outbreaks and severe cases [6]. The epidemic strains are often associated with increased production of toxins A and B, increased resistance to fluoroquinolone antibiotics, and production of binary toxin [7]. A recent advisory from the Center for Disease Control and Prevention (CDC) puts *C. difficile* on the urgent threat list, and warns of increased incidence of community acquired CDI in individuals who have not been exposed to hospital settings or antibiotic therapy. This is a population that was previously considered low risk [8]. Due to the dynamic epidemiology of *C. difficile*, it is important to phenotypically characterize both historic and current epidemic strains to define how they adapt to different environmental pressures, especially those found in the gastrointestinal (GI) tract.

Antibiotics significantly disrupt the indigenous gut microbiota, but they also alter the host and gut microbiota derived bile acids allowing for *C. difficile* colonization [9–12]. Bile acids are end products of cholesterol metabolism and essential for lipoprotein, glucose, drug, and energy metabolism [13,14]. Primary bile acids

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made by humans are cholate (CA) and chenodeoxycholate (CDCA). These bile acids are further conjugated with either taurine or glycine. Mice are slightly different, where a significant amount of CDCA is converted by the host into α -muricholate (α MCA) and β -muricholate (β MCA) [15]. Primary bile acids are released in response to food into the duodenum and a majority of them (~95%) are reabsorbed in the terminal ileum, and returned to the liver via enterohepatic recirculation. Primary bile acids that reach the large intestine (~5%) are acted upon by specific members of the gut microbiota and biotransformed via two enzymatic reactions, deconjugation and dehydroxylation, into secondary bile acids (SBAs), where they remain at relatively high concentrations (200–1000 μ M) [16]. There are approximately 50 different chemically distinct SBAs found in human large intestine [17]. Fig. 1 illustrates the most abundant SBAs: deoxycholate (DCA), lithocholate (LCA), and ursodeoxycholate (UDCA) [16]. Iso-deoxycholate (iDCA) and isolithocholate (iLCA) are 3 β -OH epimers of DCA and LCA [16,18]. Additionally, ω -muricholate (ω MCA) and hyodeoxycholate (HDCA) are only present in mice [19–21]. The physiologically relevant concentrations of SBAs in the human and mouse large intestine, and the concentrations used in the present study are presented in Table 1.

C. difficile spores require specific bile acids and amino acids for germination into metabolically active vegetative cells [22]. Primary bile acids such as taurocholate (TCA) and CA are present in high concentrations in the distal small intestine and are primary germinants for *C. difficile* spores along with glycine [16]. However, CDCA, another primary bile acid, is able to inhibit *C. difficile*

germination [23]. Recent studies show that depletion of the microbial members responsible for converting primary bile acids to SBAs reduces resistance against *C. difficile* in the large intestine [24–27]. There are studies showing that the SBAs ω MCA, LCA, UDCA, and HDCA inhibit TCA mediated spore germination in *C. difficile* UK1, M68, and VPI 10463, and growth of strains CD196, UK14, UK1, M68, and VPI 10463 *in vitro* [11,28,29]. However, there are fewer studies showing how SBAs alter all stages of the *C. difficile* life cycle (spore germination, growth, and toxin activity) of clinically relevant epidemic, non-epidemic, historic, and more recent strains.

Past studies have shown that spore germination rates of clinically relevant *C. difficile* isolates varied when exposed to primary bile acid TCA and CDCA, a known inhibitor of spore germination *in vitro* [23,30]. Therefore, it is conceivable that differences may also exist in the inhibitory effect of SBAs on not only spore germination, but on other stages of the *C. difficile* life cycle including growth, and toxin activity, which mediates disease. We hypothesize that spore germination, growth, and toxin activity of clinically diverse *C. difficile* strains will vary in their sensitivity to SBAs. Here we show the differences in the inhibitory effect of SBAs; specifically DCA, iDCA, LCA, iLCA, UDCA, ω MCA, and HDCA, on TCA mediated spore germination and outgrowth, growth, and toxin activity of clinically relevant *C. difficile* strains R20291 and CD196 (ribotype 027), M68 and CF5 (017), 630 (012), BI9 (001) and M120 (078). Defining how SBAs affect different stages of the *C. difficile* life cycle in clinically relevant strains will help us understand how different strains are able to survive and cause disease in the GI tract. It will also allow us

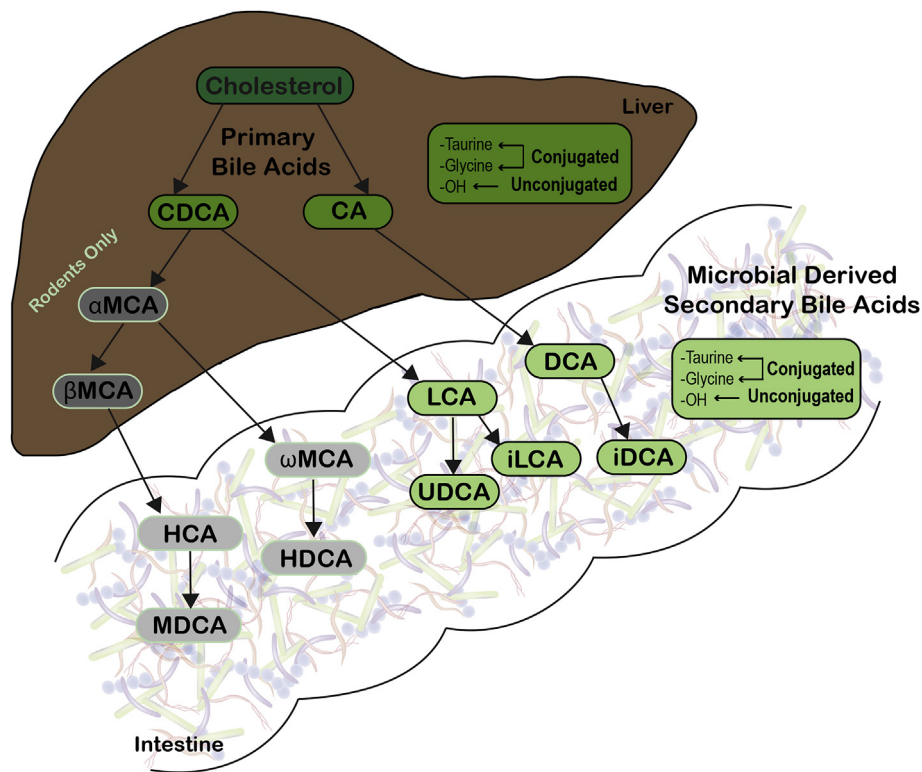


Fig. 1. Production of gut microbial derived secondary bile acids. Primary bile acids, chenodeoxycholate (CDCA) and cholate (CA) are synthesized from cholesterol by hepatocytes in humans and rodents. In rodents, a portion of CDCA is further converted into α -muricholate (α MCA) and β -muricholate (β MCA), which are not recognized in humans (represented in gray). Primary bile acids can be unconjugated or further modified via conjugation to taurine or glycine within the liver. Once synthesized, host derived primary bile acids (represented in darker shades) enter into bile. Bile is stored in the gallbladder until release in the duodenum following ingestion of a meal. Once within the gastrointestinal tract, the gut microbiota can convert host derived primary bile acids into secondary bile acids (represented in lighter shades). Microbial derived secondary bile acids can also be unconjugated or conjugated to taurine or glycine. Image modified from Winston et al., [27]. Abbreviations: CA, cholate; CDCA, chenodeoxycholate; DCA, deoxycholate; iDCA, iso-deoxycholate; HCA, hyocholate; HDCA, hyodeoxycholate; LCA, lithocholate; iLCA, iso-lithocholate; MDCA, murideoxycholate; UDCA, ursodeoxycholate; α MCA, α -muricholate; β MCA, β -muricholate; ω MCA, ω -muricholate.

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