INRS – Centre Armand-Frappier Santé Biotechnologie

CHARACTERIZATION OF THE IMPACT OF INTESTINAL MICROBIOTA AND A SPECIFIC PROBIOTIC FORMULATION ON AGR *QUORUM SENSING* AND VIRULENCE IN *CLOSTRIDIOIDES DIFFICILE*

By

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RÉSUMÉ

Clostridioides difficile (*Clostridium difficile*) est responsable des infections à *C. difficile* (ICD). Les ICD sont fortement associées aux antibiotiques qui altèrent le microbiote intestinal, menant à la dysbiose. Le *quorum sensing* (QS), une communication intercellulaire, est associé à la régulation de la virulence. L'impact potentiel du microbiote dans l'interférence du QS des pathogènes et son effet sur la virulence sont inconnus. Cette étude se voulait de caractériser le rôle du microbiote et des probiotiques dans la modulation du QS *agr* de *C. difficile.* Ce travail a démontré l'impact spécifique du microbiote, selon son état, sur la production de toxines et la régulation du QS de *C. difficile* ainsi qu'un effet potentiel d'interférence du QS de *C. difficile* associé avec la présence de probiotiques. Les résultats ont également suggéré une régulation indépendante des deux loci *agr : agr1* et *agr2.* Ce travail a contribué à la compréhension de la réponse de *C. difficile* en présence de microbiote normal, en dysbiose et supplémenté avec une formulation probiotique spécifique. Finalement, ce travail a permis de soulever les différentes limitations du modèle *ex vivo* ayant été mis en place pour cette étude et a contribué à élaborer des pistes de solution en vue d'optimiser le modèle.

Mots-clé : *Clostridioides difficile;* microbiote; antibiotiques; probiotiques; *quorum sensing*; virulence; toxine.

ABSTRACT

Clostridioides difficile (*Clostridium difficile*) is responsible for *C. difficile* infections (CDI). CDI are strongly associated with antibiotics as they alter the gut microbiota which lead to dysbiosis. *Quorum sensing* (QS), an intercellular communication system, is associated with the regulation of virulence but is not fully understood in *C. difficile*. Furthermore, the potential impact of the gut microbiota to interfere with the pathogen's QS and its subsequent effect on the virulence are unknown. This study aimed to characterize the role of the intestinal microbiota and a probiotic supplementation in modulation of *C. difficile agr* QS. This work highlighted the specific impact of the microbiota's state on *C. difficile* QS regulation and suggested a potential interfering effect on *C. difficile* QS associated with the presence of a specific probiotic formulation. Moreover, the results suggested an independent regulation of the two *agr* loci*: agr1* and *agr2.* This work has contributed to the understanding of *C. difficile* response in the presence of normal, disrupted and probiotic supplemented microbiota. Ultimately, this work has raised the limitations within the *ex vivo* model put in place for this study and has contributed to elaborate solution pathways with the purpose the model's optimisation.

Key words: *Clostridioides difficile;* microbiota; antibiotics; probiotics; *quorum sensing*; virulence; toxin.

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ABBREVIATIONS

Agr	Accessory gene regulator
AGCC	Acide gras à chaînes courtes (fr)
AHL	*N*-acyl homoserine lactones
AI	Autoinducer
AIP	Autoinducer peptide
ANOVA	Analysis of variance
CD	*Clostridioides difficile*
CDI	*C. difficile* infection
cDNA	Complementary DNA
CFU	Colony-forming unit
Cq	Quantification cycle
ELISA	Enzyme-linked immunosorbent assay
g/L	Grams per litre
GIT	Gastrointestinal tract
GR	Gène de reference (fr)
h	Hour
hrs	Hours
ICD	Infection à *C. difficile* (fr)
mg	Milligrams
mg/L	Milligrams per litre
min	Minute
mL	Millitre
mM	Millimolar
ng	Nanogram
nm	Nanometre

NRT	No reverse transcriptase control
NTC	No template control
OD	Optical density
PaLoc	Pathogenecity locus
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
QS	*Quorum sensing*
RGs	Reference genes
RT-qPCR	Reverse transcription quantitative PCR
s	Second
SEM	Standard deviation of the mean
TY	Tryptose Yeast Extract
TMF	Transplantation de microbiote fécal (fr)
UV	Ultraviolet
V	Voltage
vol	Volume
w	Weight
w/vol	Ratio weight/volume
×*g*	Relative centrifugal force
°C	Degree celsius
µg	Microgram
µL	Microlitre
µm	Micrometre

1 INTRODUCTION

1.1 Clostridium genus

Clostridium genus is one of the largest prokaryotic genera, containing Gram-positive, rod shaped, anaerobic and spore-forming bacteria (Cruz-Morales et al., 2019). While the majority of the *Clostridium* species is benign, few of them are well known for their implication in biotechnology processes and emerging clinical treatment potential. Non-pathogenic specimen like *Clostridium acetobutylicum, Clostridium sporogenes* and genetically modified *Clostridium novyi* have sollicitated great interests for their role in bio-butanol production or their use in cancer therapy. Recent advances in bioengineering have open great opportunities in the use of these bacteria in industrial processes of bio fuel because of their natural capacity to produce solvents such as acetone, ethanol and butanol (J. Lee et al., 2012; Li, Huang, Ke, Pang, & Liu, 2020; Papoutsakis, 2008). Moreover, their anaerobic environment requirements make some non-pathogenic strains of *Clostridium* great potential tools for targeting tumors as those organisms will growth only where oxygen levels are very low (hypoxic) such as in necrotic tissues of solid tumors. *C. novyi-NT,* a genetically modified strain that lost the alpha-toxin gene by heat treatment, is derived from wild-type *C. novyi* and is devoid of the lethal toxin (Dang, Bettegowda, Huso, Kinzler, & Vogelstein, 2001). The combined use of *C. novyi-NT* and more commun treatment such as chemotherapy and immunotherapy has shown great preclinical results (Agrawal et al., 2004; Dang et al., 2004). Genetically engineered *C. sporogenes* harboring *Escherichia coli* cytosine deaminase enzyme was able to convert a non toxic prodrug into a toxic anticancer drug which result in significant antitumor activity *in vitro* and *in vivo* (Liu et al., 2008; Liu, Minton, Giaccia, & Brown, 2002).

Despite the beneficial roles of few of the *Clostridium* representatives, pathogenic *Clostridium* species are responsible of numerous major health problems in humans and animals and continue to overshadow the “good ones”. These species cause disease by the release of various toxins, including neurotoxins by *C. botulinum* (A. T. Carter & Peck, 2015) and *C. tetani* (Chapeton-Montes et al., 2020; Schiavo et al., 1992), and enterotoxins/cytotoxins by *C. perfringens* (Navarro et al., 2018; Shrestha et al., 2018) and *C. difficile* (Chandrasekaran & Lacy, 2017; Rolfe & Finegold, 1979).

1.2 Clostridioides difficile

Clostridioides difficile (previously known as *Clostridium difficile*) is an opportunistic pathogenic bacterium better known as the causative agent of *C. difficile* infection (CDI) that presents itself usually as *C. difficile*-associated diarrhea (CDAD) and pseudomembranous colitis (CDAC). The pathogen, initially named *Bacillus difficilis,* was first discovered and characterized by Hall and Toole in 1935 but its association with antibiotic-associated diarrhea (AAD) was documented only in 1978 (George, 1978; Larson, 1978). Nowadays, *C. difficile* is recognized as one of the most important nosocomial pathogens.

1.3 *C. difficile* infections

In the early 2000's era, CDI have been responsible for multiple large outbreaks in North America, which have been rapidly spreading around the globe. This rapid increase in cases have been attributed to the then-emerging NAP1/027/BI strain. This strain showed new resistance to fluoroquinolones, which was in accordance with the widespread use of these antibiotics during the same period. Moreover, better diagnostic techniques and a greater attention from physicians must have participated in higher number of CDI cases (Moehring, Lofgren, & Anderson, 2013).

CDI is acquired by ingestion of spores of *C. difficile* transmitted by the fecal-oral route. Spores can come from multitude biological sources such as asymptomatic carriers, infected patients and animal gastrointestinal tracts (GIT) but also from contaminated environment where spores can settle on hard surfaces and be transported by patients and personnel, which is a major problem in hospital settings. Spores are resistant to heat, antibiotics and the majority of disinfecting agents. Moreover, because spores are not affected by the acidic pH of the stomach, they continue their route into the GIT and reach the intestines. Germination of spores is then facilited in the small intestine due to altered ratio of primary and secondary bile acids, the latter acting as germinants. The metabolically active vegetative cells will then colonize the host by occupying a vacant niche into the distal intestines, more than often following an antibiotic-associated microbiota disruption and start producing its toxins which will leads to diarrhea (Figure 1). CDI represent approximately one-third of antibiotic-associated infections (Magill et al., 2014; Simor, 2010).

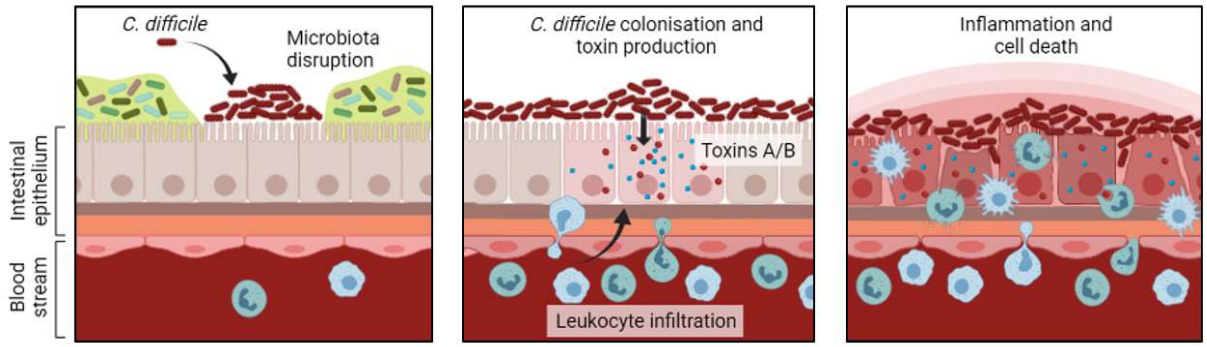

Figure 1. *C. difficile* colonisation and pathogenesis in a disrupted gut environment.

Figure inspired by Hookman & Barkin (2009).

CDI causes more usually watery than bloody diarrhea that appears within 2 or 3 days, but can be delayed to few months depending on the administration of antimicrobial agents or be absent in certain cases (Nwachuku et al., 2020). Severity of CDI is quite broad ranging with symptoms going from mild to severe symptoms that usually included diarrhea (CDAD) and less commonly, colitis (CDAC). Those can worsen to more severe and life-threatening complications such as pseudomembranous colitis, toxic megacolon, septicemia and death. Loose stools, abdominal distension and/or leukocytosis are part of an early diagnosis (Hookman & Barkin, 2009). Unusual manifestations of CDI have also been reported like ascites, peripherial edema and hypoalbuminemia but also other extra-colonic manifestations like arthritis, cellulitis and necrotizing fasciitis (Birnbaum, Bartlett, & Gelber, 2008; Dansinger et al., 1996). Recurrence (rCDI) is not rare as 10-30% of CDI patients relapse a first time and those rates can reach 45-65% with subsequent relapses. rCDI is generally associated with prolonged hospital stay, antibiotic use, age over 65 years-old, comorbid medical conditions and anti-acid medication (Finn et al., 2021; Song & Kim, 2019).

C. difficile is an opportunistic pathogen that can infect health deficient hosts such as patients undergoing antibiotic treatment. *C. difficile* is also known to colonize individuals without causing the disease (absence of symptoms of infection) which is termed asymptomatic colonization and is relatively common in neonates and in the 2 first years of life varying between 15 to 70% (Al-Jumaili, Shibley, Lishman, & Record, 1984; Collignon et al., 1993; Jangi & Lamont). In 2018, asymptomatic carriage was estimated to range between 4-15% in healthy adults (Crobach et al., 2018). In acute care hospitals, asymptomatic colonization can go up to 18% of patients and 20%

for long-term care residents (Donskey, Kundrapu, & Deshpande, 2015; Simor et al., 2002). Nevertheless, those numbers can be much higher during outbreaks (Ofori et al., 2018).

In addition to cause deaths, management and treatment of CDI is costly, and represent a great financial burden (Dubberke et al., 2008). Alone, CDAD readmissions are estimanted to cost a minimum of 128,200 $ annually per facility in Canada (M. A. Miller et al., 2002).

1.3.1 Epidemiology

C. difficile infections represent a major global health problem with various current epidemic strains in North America, Europe, Middle East and Asia. Since 2005, North American pulsed-field 1 (NAP1), also designated PCR ribotype 027 (RT 027), is frequently detected in CDI patients in North America and Europe and has been associated with large outbreaks. In addition to a new fluroquinolones resistance, this strain produces a third toxin, the binary toxin and harbors a mutated *tcdC* gene which results in an appreciable increase in toxin A and toxin B production. These characteristics make the RT 027 strain more virulent and leads to his association with higher morbidity and recurrence rate (MacCannell et al., 2006; Pépin et al., 2005; Pepin et al., 2005; Warny et al., 2005). In 2011, *C. difficile* has been the causative agent of more than 450,000 infections and was associated with approximately 29,000 deaths in the United States (Lessa et al., 2015). In the ≥65 years old age group alone, incidence was 627.7 per 100,000 population which was 4 times higher than 45-64 years age group and almost 9 times higher than people aged 44 or less (Lessa et al., 2015). NAP1 strain was also associated with elevated numbers of community associated cases. Last updates show an important increase in community-associated CDI going from 170,000 cases in 2011 to 226,400 cases in 2017 in the United States (Guh et al., 2020).

Despite that high mortality rates associated with CDI remain a revelant issue nowadays, important improvements in management of CDI cases, awareness surrounding the use of broad-spectrum antibiotics and other initiatives such as antibiotic stewardship may have proven their efficacy in the control of the disease following the recent outbreaks. In a recent report by the Canadian Nosocomial Infection Surveillance Program, it is mentioned that the national rate of healthcare associated CDI shows a relative decrease of 35.8% in 2015. They also noted significant reduction in *C. difficile* NAP1 (RT 027) strain and significant increases in NAP4 and NAP11 strains. These strains were the most prevalent NAP types with 37.6%, 14.2% and 5.9% respectively and corresponding all together to more than half of the 2690 isolates tested during this period (Katz et al., 2018). The most recent published data from the Centers for Disease Control and Prevention

(CDC) report that health care-associated CDI and CDI related hospitalizations in the United States decreased by 36% and 24% respectively from 2011 to 2017 (Guh et al., 2020). Similar to the trend in Canada, ribotype 027 decreased significantly from 2011 through 2017 in the US. It has been proposed that the widespread decrease in fluoroquinolone use could have contributed to that decline (Guh et al., 2020).

1.3.2 Risk factors

Key risk factors associated with the development of CDI include antibiotic exposure, older age (>65 years-old) and hospitalization (Czepiel et al., 2019; Pépin et al., 2005). Most of CDI cases occur in elderly patients which is demonstrated by a 5-10-fold increased risk for CDI in patients aged 65 years or more. Also, older age is a significant risk to complication and increased severity and mortality (Czepiel et al., 2019; Leffler & Lamont, 2015). Incidence of *C. difficile* colonization (symptomatic or not) in the first day of hospitalization ranges between 2.1-20% and correlates positively with the length of stay, where it can reach up to 50% after 1 month or less of hospitalization (Czepiel et al., 2019).

Antibiotic consumption is considered the most important risk factor for the development of CDAD (Hookman & Barkin, 2009). Healthy immunocompetent persons are usually not permissive to *C. difficile* colonization because the pathogen fails to proliferate in the GIT following germination and likely, is unable to express virulence factors (*i.e.,* toxins) (Lawley & Walker, 2013). *C. difficile* proliferation is facilitated by disruption of the normal intestinal microbiota. Antibiotic administration represents the greater risk factor to the development of CDI as it has been associated with a dramatically decreased level of intestinal microbiota diversity and important structural changes in taxa composition (Lagier, 2016; Robinson & Young, 2010; Theriot et al., 2014). Antibiotic administration has resulted in alteration of the gut microbiota in a variety of animal models (Best et al., 2012; Chen et al., 2008; Robinson & Young, 2010; Theriot et al., 2014) and in humans (Ghimire et al., 2020). The most frequently implicated antibiotics in CDI are clindamycin, cephalosporin and fluoroquinolones (Gerding, 2004; Lagier, 2016; Owens et al., 2008; Schäffler & Breitrück, 2018).

1.3.3 Treatment

Despite the fact that antibiotics consumption is strongly associated with the infection development, antibiotics are still used for CDI treatment. Recently, fidaxomicin has been considered as the preferred treatment for initial CDI episode, but antibiotics such as vancomycin

and metronidazole (for nonsevere CDI) are still acceptable alternatives because of their higher disponibility and lower cost compared to fidaxomicine. Severe or fulminant courses are treated with higher dose of the same antibiotics, combination and/or alternative administration routes (*i.e.,* by nasograstric tube, rectally or intravenous). In addition of the antibiotherapy, the fecal microbiota transplant (FMT) therapy, a relatively new practice in the world of modern medicine, has been included as treatment for second and multiple recurrent episodes of CDI (Johnson et al., 2021; Kukla et al., 2020).

1.4 *C. difficile* virulence factors

Both *C. difficile* toxins, TcdA and TcdB, are recognised as the primary cause of CDI manisfestation in infected patients as *tcdA* and *tcdB* null mutants do not cause disease in animal models. However, other virulence such as motility contribute to the virulence of the bacterium. Additionnaly, its ability to sporulate acts as an important persistence factor and ensures its survival in the harsh conditions of the upper GIT and promotes its dispersion in the hospital environment contributing to its transmission between hosts. Other virulence factors such as adhesins and antibiotic resistance have also great impact in allowing *C. difficile* expansion into the gut following antibiotic disruption (Awad et al., 2014). These factors greatly contribute to the emergence of epidemic strains worldwide.

1.4.1 *C. difficile* toxins

Toxin A (TcdA) and toxin B (TcdB), whose genes are located among others on a 19.6 kb pathogenicity locus (PaLoc), are responsible for CDI symptoms (Awad et al., 2014; Smits et al., 2016). The toxin genes are framed by three accessory genes; *tcdC* and *tcdR* that are implicated in regulation and *tcdE,* implicated in secretion. A third toxin, the binary toxin (CDT) has been identified in 23% of virulent strains such as RT 027 and 078 (Bauer et al., 2011). CDT has been demonstrated to enhance virulence of those strains (Cowardin et al., 2016; Schwan et al., 2009). CDT is an actin-ADP-ribosyltransferase and is encoded by *cdtA* and *cdtB* genes located on a second locus named Cdt locus (or CdtLoc) (Figure 2).

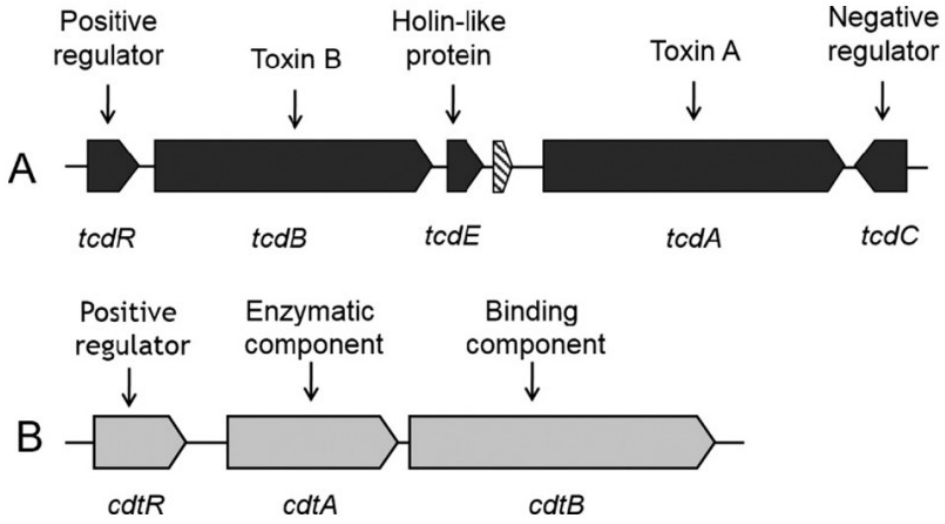

Figure 2. The two main toxin loci of *C. difficile*.

The PaLoc which encodes the toxin A and toxin B, as well as three accessory proteins: TcdR, TcdE and TcdC. A partial pseudogene is present downstream of *tcdE*, shown with a dashed fill. (B) The CdtLoc which encodes the two binary toxin genes and one accessory gene. Figure taken from Elliott *et al.* (2017).

Following the expansion of *C. difficile* in the GIT and the release of toxins, TcdA and TcdB enter epithelial cells by endocytose. After vesicular acidification of the endosome and pore formation, the toxins translocate into the cytosol where the glucosyltransferase domain (GTD) of the toxins is cleaved and released (Orrell et al., 2017; Reineke et al., 2007). The glucosyltransferase activity of the toxins inactivate specific host Rho and Ras GTPases through glycolysation and leads to further disruption of host cell functions (Chandrasekaran & Lacy, 2017). Both toxins show cytotoxic effects on a wide variety of cell types *in vitro* described as retraction of cell processes and cell rounding (C Pothoulakis, 1986; Taylor et al., 1981; Wedel et al., 1983). As reviewed by Chandrasekaran and Lacy (2017), translocation of toxins into the colonic epithelial cells leads to cytopathic and cytotoxic effects including cell death, disruption of epithelial tight junctions and epithelial integrity, increased mucosal permeability and fluid secretion, and acute inflammatory response through induced pro-inflammatory cytokines production, neutrophil recruitment and tissue damage.

While the hypervirulent strain RT 027 and many other epidemic strains have shown to secrete both toxin A and toxin B, studies support the concept that toxin B is in fact, contributing in a greater manner to severity of CDI than toxin A. Toxin B alone was sufficient to induce disease in hamsters. Administration of *C. difficile* mutants A^-B^+ to hamsters that had received clindamycin

resulted in similar CDI symptoms, mortality rate and histopathological damages than the wild-type strain A^+B^+ (Lyras et al., 2009). In Asian countries, RT 017 (A^-B^+ mutant) is one of the most prevalent strains and has been associated with CDI outbreaks and higher 30-day mortality in patients with comorbidities (Kim, Kim, & Pai, 2016). In 2015, a clinical isolate from a patient with diarrhea was found to have a complete deletion of *tcdA* and *tcdC* genes and has no binary CDT genes (Janezic, Marín, Martín, & Rupnik, 2015). In the light of those results and the emergence of clinical isolates producing only the toxin B, TcdB has recently been described as the primary virulence determinant for CDI (Bilverstone et al., 2020).

Both A^+B^- and A^-B^+ variants have been isolated from humans (Monot et al., 2015). In the hamster CDI model, infection with mutant A^+B^- strain showed great expansion of the bacteria in the gut and was associated with mild ceacal histologic damage (Marvaud, Quevedo-Torres, Eckert, Janoir, & Barbut, 2019). However, in this study, A^+B^- strain did not cause mortality or clinical signs which was in contradiction with previous reports (G. P. Carter et al., 2015; Kuehne et al., 2010).

1.5 Implication of *Quorum sensing* in pathogenic bacteria virulence

Quorum sensing (QS) can be described as an intercellular communication and more often designated as *cell-to-cell* communication. In the prokaryotic world, this kind of communication is possible by the release in the environment of molecules called autoinducers (AI) by bacteria. AI accumulation in the environement is function of the population growth. When the community grows bigger, AI concentration reachs a threshold and subsequent modulation of target gene expression follows (de Kievit & Iglewski, 2000). The discovery that bacteria have the capacity to communicate shedded light onto the concept of coordination. It is now known that bacteria can act as a collectivity, sensing the density of bacteria in a defined environment and subsquently coordinate various cellular mechanisms simultaneously in their best interests. The most pertinent function of *quorum sensing* is the coordination of virulence factors by the bacterial population in the context of infection. In some bacteria, toxin production mechanism is known to be under *quorum sensing* regulation. As the production of toxins is energy consuming, it is in the best interest of a bacterial community to have sufficient amount of members to ensure the success of the infection. Other mechanisms such as mobility, sporulation and formation of biofilm have been associated with QS.

Three systems have been described so far, two of which are intraspecies communication and differ between Gram-negative and Gram-positive bacteria. The vast majority of Gram-negative utilizes small signaling molecules: *N*-acyl homoserine lactones (AHL) (de Kievit & Iglewski, 2000). The *las* and *rhl* QS systems of *Pseudomonas aeruginosa,* an opportunistic pathogen, are among the best-known QS systems implicated in pathogenicity. Many of *P. aeruginosa* extracellular virulence factors are regulated in a cell-density manner and the implication of QS in virulence of *P. aeruginosa* has been confirmed in various animal models (Deep et al., 2011). The signal molecules used by Gram-positive bacteria differ from AHLs used by Gram negative bacteria. Post-translationnaly processed peptides act as AI in Gram-positive QS systems. Those systems are explored in the next section.

Finally, a third QS system has been discovered and subsequently found in almost all bacteria investigated, it is the LuxS/AI-2 system. LuxS catalyses the formation of a third class of AI molecule, AI-2, a furanosyl borate diester derived from 4,5-dihydroxy2,3-pentadione (DPD). LuxS is part of the methionine metabolism (Winzer & Hardie, 2003). Putative LuxS have been found in almost all Gram-negative and Gram-positive bacteria tested (Bassler et al., 1997). Since they found that a number of bacteria produced an AI-2-like activity, Bassler *et al.* (1997) suggested that the lower-specificity LuxS QS system is used as a general interspecies communication. The accumulation of AI-2 in the supernatant of bacterial cultures has been shown to be transient as the molecule concentration will increases during exponential growth and reaches its maximum in late exponential-phase. After entry into stationary phase, levels of AI-2 decrease rapidly (Winzer & Hardie, 2003).

Since its discovery, implication of AI-2 in human pathogens virulence has been demonstrated in various activities such as biofilm production, mobility and toxin production (Elvers & Park, 2002; Girón et al., 2002; Hammer & Bassler, 2003; Ohtani et al., 2002).

1.5.1 *Quorum sensing* in Gram-positive bacteria

Gram-positive bacteria use peptides as signal molecules. The pro-AI peptide is translated and released in the cytosol. The precursor is then processed and simultaneously transported across the membrane and released in the environment. The mature peptide (AIP) then binds to a histidine kinase sensor at the surface of the cell which triggers its autophosphorylation. Interaction of the sensor with the AIP activates a phosphorylation cascade and leads to the phosphorylation of a regulator which then leads to the modulation of targets genes through a two-component signal transduction system (de Kievit & Iglewski, 2000). QS system of *Staphylococcus aureus,* the

accessory gene regulator (*agr)* system is certainly one of the best understood in Gram-positive bacteria (Figure 3). *S. aureus* QS systems have been extensly reviewed (de Kievit & Iglewski, 2000; Rutherford & Bassler, 2012). The *agr* locus comprises two operons, RNAII and RNAIII. The first contains four genes called *agrBDCA.* AgrD and AgrB are responsible for the generation of the pro-AI and the process/release of the AI respectively. AgrC encodes for a signal transducer and acts as the AIP sensor at the surface of the cell. AgrA is a response regulator of the LytTR family. Phosphyrylation of AgrA stimulates transcription of RNAIII, the latter upregulating the *agr* locus. In a context of early infection, *S. aureus* cells establish a biofilm to protect the bacteria against the host which allows the community to reach high cell density and AIP accumulation. At the point where the bacterial community has reached high density, the QS system regulated cascade induces the switch from a state of dormant cells (inhibition of biofilm formation) and early infection (down regulation of surface virulence factors) to a more active infection state with the upregulation of secreted virulence factors such as α-toxin (Rutherford & Bassler, 2012).

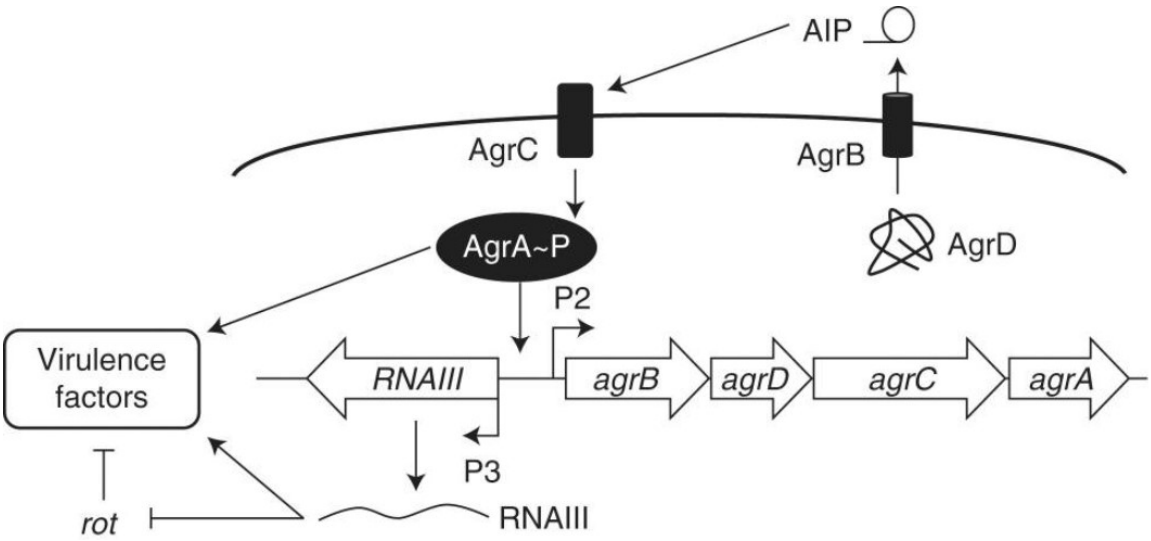

Figure 3. *S. aureus* Agr QS circuit.

The autoinducer peptide (AIP) is synthesized as a precusor from *agrD.* The AIP is then processed and released in the extracellular space by AgrB. AIPs accumulate outside the cells as a function of cell density. After reaching a threshold, AIPs are detected by the two-component signal transduction pathways involving the membrane-bound histidine kinase AgrC and the reponse regulator AgrA. Phosphorylated AgrA activates the P2 and P3 promoters encoding the *agr* operon RNAII and the regularoty RNA called RNAIII. RNAIII post-transcriptionally actives virulence factors production and represses expression of *rot,* the toxins repressor, which leads to further de-repression of virulence factors. Figure taken from Rutherford and Bassler (2012).

Regulation of toxigenesis and sporulation in *Clostridia* by *agr*-mediated signaling systems have been demonstrated in clinically revelant pathogens such as *C. botulinum* (Cooksley et al.,

2010), *C. perfringens* (J. Chen et al., 2011) and *C. difficile* (Darkoh et al., 2015; M. J. Martin et al., 2013).

1.5.2 Quorum sensing systems in *C. difficile*

The complete *agr* locus has been identified in the genome of toxigenic *C. difficile* R20291 strain (RT 027) and other clinical isolates (Melissa J. Martin et al., 2013; Stabler et al., 2009). *C. difficile* 630 genome, in the other hand, only encodes a partial *agr* locus containing only *agrD* and *agrB* genes (Stabler et al., 2009). The *agr* locus of *C. difficile* R20291 shows similiarities with the *agr* locus of *S. aureus* but order of genes is inverted (Figure 4). *C. difficile*'s AgrA, AgrC and AgrB share 28, 23 and 25% amino acid identity with the equivalent *S. aureus* proteins respectively. AgrD, the AIP precurseur, shares no significant similarity with *S. aureus* (Melissa J. Martin et al., 2013).

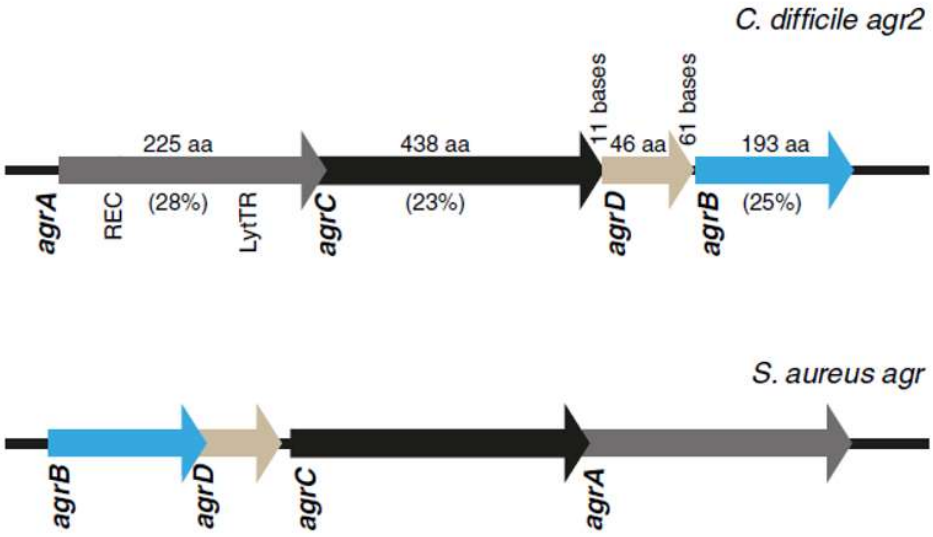

Figure 4. Genetic organization of *agr2* locus in *C. difficile* R20291 and *agr* locus in *S. aureus.*

Arrows indicate genes and direction of transcription. The length of each protein is indicated above (aa, amino acids), and the percent identity to the respective *S. aureus* orthologue is indicated below. Figure taken and adapted from Martin *et al.* (2013).

1.5.3 Virulence regulation by the *agr* system in *C. difficile*

Investigation of the regulatory network of *C. difficile agr* systems demonstrated that AgrA is implicated in the regulation of flagellar biosynthesis genes and *tcdA,* c-di-GMP regulatory protein genes and two-component regulatory systems (Melissa J. Martin et al., 2013). RNA sequencing analysis showed a repression of flagellar biosynthesis genes as well as an under expression of *tcdA*

gene in *agrA C. difficile* mutant. Reduced production of toxin A *in vitro* confirmed the previous observation. This group also demonstrated that the insertional inactivation of *agrA* causes underexpression of *agrC* and suggested that *agrAC* forms a single transcriptional unit and that the complete *agr* locus (*agrBDAC)* is potentially a single transcript like it is demonstrated in *S. aureus* (Melissa J. Martin et al., 2013).

Roles of *agrBD* in pathogenesis of *C. difficile* have been further characterized with the creation of mutants with complete deletion of *agrB1D1 (agr1* locus) or *agrB2D2* (*agr2* locus) by allelic exchange (Darkoh et al., 2016). This group has demonstrated that *agr1* locus is essential to the virulence of the pathogen. In fact, *agrB1D1* mutant showed abolishment of *tcdA* and *tcdB* transcripts levels and toxin activity was suppressed. On the opposite, there was no difference in expression level of toxins transcripts neither toxin activity in the *agrB2D2* mutant compared with the parental strain. As it was previously proposed, AgrD1 is responsible for the generation of the pro-AIP and is required to activate toxin production (Darkoh et al., 2015; Darkoh et al., 2016). Moreover, while capable to colonize susceptible mice, no toxins were detected in fecal sample of mice infected with the *agrB1D1* null mutant. Infection with the *agrB1D1* mutant resulted in a survival rate of 92% compared to 8% when mice were infection with the wild-type strain.

1.5.4 Virulence regulation by the LuxS/AI-2 system

Implication of LuxS/AI-2 system in virulence in *C. difficile* have been discussed in few reports. Some studies have concluded that LuxS system indeed plays a role in modulation of virulence factors such as a positive regulation of *tcdA, tcdB* and *tcdE* transcript and biofilm formation (Đapa et al., 2013; A. S. Lee & Song, 2005; Slater et al., 2019). However, opposite opinions suggested that AI-2 represents merely a metabolic by-product of the methyl cycle and that its transport outside the cells would be associated with its potential toxicity (Glen P Carter et al., 2005). These investigators showed that AI-2 was not involved in regulation of toxin production. They further highlighted how it is not obvious that AI-2 could possibly coordinate toxin production in *C. difficile* because toxin accumulation occurs in the stationary phase, while AI-2 concentration reachs its maximum in late exponential phase and rapidly decreases when entering stationary phase.

Few years after, Patel's work demonstrated that supernatant from *luxS* mutant showed similar cytotoxicity than the wild-type strain on vero and HT29 cells, which would support the idea that AI-2 is not directly involved in the regulation of toxin A or B in this species. In addition, inactivation of *luxS* did not affect sporulation nor germination (Patel, 2011).

While strong evidence have been reported describing the implication of *agr* system in the virulence of *C. difficile,* the role of LuxS/AI-2 system in *C. difficile* virulence is still not elucided and fully understood to date. Nonetheless, this signaling system is indeed involved in various other pathogen functions associated with virulence as reviewed by Pereira et al. (2013).

1.6 The capacity of intestinal microbiota to prevent pathogen invasion

1.6.1 Concept of colonization resistance

Colonic microbiota is one of the largest pool of microorganisms in mammals. Five main phyla can be found in the gut; *Firmicutes* and *Bacteroidetes* who collectively represent 90% of the microbiota followed by *Actinobacteria, Proteobacteria* and *Verrucomicrobia* (Arumugam et al., 2011; Backhed et al., 2005; Rinninella et al., 2019). The microbiota is a large balanced microbial ecosystem that plays a central role in the host development and basic physiology as well as protecting the host against pathogens, which can be designated by the colonization resistance.

Lawley and Walker (2013) defined the intestinal colonization resistance as "the resistance to colonization by ingested bacteria or inhibition of overgrowth of resident bacteria normally present at low levels within the intestinal tract". They further described a working model of colonization resistance as a multiple layers of host defense. They stated that these layers arise from direct and indirect mechanisms and interactions between the invading pathogen, intrinsic microbiota and the host immune system (Lawley & Walker, 2013). Those interactions have evolved closely so that each component of these interactions may benefit from it. This close relationship has enabled the formation of homeostatis and health preservation.

Multiple "layers of host defense" - or mechanisms - use by the microbiota have been described: competitive exclusion through nutriment-niches availability (Girinathan et al., 2020; Jenior, Leslie, Young, & Schloss, 2017; Litvak & Baumler, 2019), exclusion by short-chain fatty acids (SCFA) production (Fachi et al., 2019; Louis & Flint, 2017; Parada Venegas et al., 2019); bile salt metabolism (Nagao-Kitamoto et al., 2020; Seekatz et al., 2018; Theriot et al., 2016) and production of inhibitory molecules (Khattab et al., 2020; Rea et al., 2010).

The loss of colonization resistance has been associated with intestinal microbiota disruption, designated as "gut dysbiosis".

1.6.2 Gut dysbiosis

The term dysbiosis is more than often defined as an “imbalance of the microbiota” from which “imbalance” being defined as a loss of homeostasis in contemporary literature (Hooks & O'Malley, 2017). The loss of homeostasis is further characterized by various traits in context of gut microbiome, including reduced microbiota diversity, thinner mucus layer, increased epithelial damages of the mucosa, and accentuated inflammation (immune cells influx, increased AMPs production and targeted Igs) (Vonaesch et al., 2018).

Implication of gut dysbiosis as a causative agent in various disease is increasingly recognized and has been associated with increased severity in intestinal diseases such as irritable bowel syndrome (IBS), inflammatory bowel diseases (IBD) including ulcerative colitis (UC) and Crohn’s disease (CRD), colorectal cancer, type-2 diabetes and obesity but also in neuropsychiatric disorder such as autism (Amabebe et al., 2020; Ebrahimzadeh Leylabadlo et al., 2020; Gagniere et al., 2016; Menees & Chey, 2018; Mentella et al., 2020; Pulikkan et al., 2019).

In the midst of the 20th century, indigenous intestinal microbiota was first associated with the concept of colonization resistance to pathogens when it was demonstrated that antibiotic treatment leads to a 100,000 fold decrease in the dose of *Salmonella enterica* serovar Typhimurium needed to infect mice (C. P. Miller et al., 1956). In the 1980s, interest increased in the concept of colonization resistance of the normal gut microbiota against the then-newly found agent of pseudomembranous colitis, *C. difficile.* Those colitis were associated with antibiotic administration in humans (Bartlett, 1979). It was then demonstrated that healthy and undisturbed (normal) mice cecal microbiota exhibited colonization resistance against *C. difficile* and that antibiotic disturbance is necessary for proliferation and toxin production (Borriello & Barclay, 1986).

More recent work supports this concept as it was demonstrated that inoculation of toxigenic *C. difficile* vegetative cells or spores in emulsion of cecal content from control mice do not results in overgrowth or toxin production. In contrast, cecal samples from mice treated with antibiotics permitted *C. difficile* growth and toxin production (Adams, Riggs, & Donskey, 2007). In accordance with previous results, another group used a triple-stage chemostat model of the human gut to investigate *C. difficile* proliferation and toxin production following a treatment with cefotaxime. Proliferation of *C. difficile* was only observed when there was an exposure to the antibiotics and was followed by toxin production (Freeman, O’Neill, & Wilcox, 2003).

Antibiotics are known to alter the microbial community and lead to disruption of the microbiota, decrease in bacterial richness and diversity and alteration of metabolome. Since

disruption of the microbiota and its re-establishment is associated with pathogen clearance (Endt et al., 2010) and resolution of symptoms (Brandt et al., 2012; Dowle, 2016), the intrinsic capacity of colonization resistance of the gut microbiota has been an important concept guiding development of therapies for preventing pathogenic colonization and infections.

1.7 Alternative therapies for the prevention and treatment of CDI

The fast accumulation of large epidemic outbreaks around the globe by NAP1 strain has been associated with its increased antimicrobial resistance. It has been demonstrated that NAP1 has reduced susceptibility to metronidazole, rifampicin, clindamycin, chloramphenicol, moxifloxacin and imipenem (Krutova et al., 2018) . The fact that antibiotics are still first line therapy to treat CDI which are caused by a multi-resistance pathogen, underlies the critical need for alternatives approaches for the treatment of the disease.

Nowadays there is a race to find and introduce alternatives to antibiotics to treat CDI and to better prevent the disease. Alternatives treatments currently studied include fecal microbiota transplant (FMT), vaccines (A. A.-O. Cox et al., 2020; Mathur, Rea, Cotter, Ross, & Hill, 2014), antimicrobioals (Speri et al., 2020) and probiotics. While vaccines and antimicrobials research are in their early stages, FMT brought a lot of interest in the last few years. While being relatively new in the modern medical field, FMT has been a proven strategy to treat recurrent CDI patients. FMT is the administration of a gut microbiota isolated from a healthy donor to a dysbiotic patient. Even with rising concerns about the possible effects in recipients (Paramsothy et al., 2017), this method is increasingly used in the treatment of CDI patients who experienced recurrent infections and do not respond to antibiotic treatment. FMT has also been proposed as a potential treatment in primary CDI despite opposing opinions and guidelines (Juul et al., 2018; Merrick et al., 2020; Mullish et al., 2018). While FMT has recently been considered as safe and effective in the medium- to long-term with the maintains of the transferred healthy microbiota up to 24 months post-FMT (Barberio et al., 2020), CDI still reoccurs in 10% to 15% of recipient following FMT failure (Tariq et al., 2020). Furthermore, FMT represents a multi-step treatment beginning with the stool donor recruitment and screening. Because of huge logistics involved in donors managing and because a notable portion of potential donors are deferred at each step of sample screening, this process is costly and time-consuming and can lead to importants delays in receiving treatment. In a recent study, only a lean 1.7% (134/ 7,968) of prospective donors were qualified for stool donation (Dubois et al., 2020). Finally there are great concerns about disease transmission to the recipient

from biological material from donors because these uncharacterized microbial populations can contain both protective and harmful microbials (Giles et al., 2019). All these concerns motivate experts to seek alternative approaches such as the use of well caractherized specific microbiota preparations like probiotics.

1.7.1 Probiotic supplementation

Probiotics have been proposed and investigated as a prophylaxis strategy and in some case, a potential treatment of primary and recurrent CDIs. Compared to FMT, a known composition of microbial supplement would ensure its safety when throughfully characterized and validated. Secondly, processing and delays in receiving treatment would be shortened. Third, probiotic supplementation represents a cost-effective solution compared to traditional treatments (Leal et al., 2016). Health claims among commercialy available probiotics may include supporting digestive health or bowel function, promoting healthy digestion, maintains of digestive balance of normal and healthy intestinal flora, weight managing, treatment and/or prevention of diarrhea, constipation, ulcerative colitis, symptoms of IBS, and more (Sniffen et al., 2018).

1.7.2 Mechanisms of action of probiotics

Specific probiotic strains are known for their ability to effectively colonize and use diverse niches into the GIT where they can exercise multiple anti-pathogens and physiologics functions. Probiotics' mechanisms of action include the production of inhibitory substances such as bacteriocins, and SCFAs and the induction of defensins release from epithelial cells (Flynn et al., 2002; Guinane et al., 2016; Ng et al., 2009; Tabasco et al., 2009), competitive exclusion through inhibition of adhesion and translocation (Mack et al., 1999; Pothoulakis et al., 1993), utilization of nutrients and participation in the metabolism of bile acids, modulation of the immune system and improvement of the barrier function (Parassol et al., 2005; Qin et al., 2005; Yan et al., 2007), which includes increasing the mucin production (Caballero-Franco, Keller K Fau - De Simone, De Simone C Fau - Chadee, & Chadee).

1.7.3 The use of probiotics to prevent and treat CDI

The principal objective in the use of probiotics is either to restore unbalanced microbiota or support the existing healthy microbiota, improving general heath. Its use can also be recommended for the prevention or the treatment of specific GIT diseases and/or symptoms. Probiotics are by definition, "live microorganisms which when consumed in adequate amounts confer a health

benefit to the host" (Hill et al., 2014). The most studied probiotic strains are representatives of the genera *Lactobacillus*, *Bifidobacteria* spp. and *Saccharomyces.* The appreciable interest in the use of probiotics in treating and preventing CDI arises from the idea that since microbiota disruption and abolishment of colonization resistance precede CDI, probiotics would support normal indigenous flora, enhance its anti-pathogen activity (i.e., colonization resistance) by direct and indirect mechanisms and prevent further disruption and *C. difficile* expansion and/or pathogenesis.

Efficacies of various probiotics formulations in the prevention of CDI have been studied in clinical trials, including *Saccharomyces boulardii, Lactobacillus rhamnosus GG* and *Lactobacillus plantarum* 299v and probiotics mixtures like *Lactobacillus casei* DN-114 001 (*Lactobacillus casei imunitas*), *Streptococcus thermophilus,* and *Lactobacillus bulgaricus* mix (Actimel, Danone, France), *Lactobacillus acidophilus* CL1285, *Lacticaseibacillus casei* LBC80R et *Lacticaseibacillus rhamnosus* CLR2 (Bio-K Plus, a Kerry company, Canada) and *Lactobacillus acidophilus* NCFM, *Lactobacillus paracasei* Lpc-37, *Bifidobacterium lactis* Bi-07 and *B. lactis* Bl-04 mix (Mills et al., 2018; Na & Kelly, 2011). While it seems that few specific probiotics show efficacy in reducing CDI incidence and symptoms, credibility of numerous clinical trials have been questioned because of small study population, conflicting results, selection criteria biais, insufficient blinding and high heterogeneity. Larger and well-controlled studies are still necessary to confirm those positive findings and to determine the real potential of probiotics in preventing CDI (Mills et al., 2018; Na & Kelly, 2011).

1.8 Hypothesis and objectives of this study

The hypotheses of this study were:

- A healthy and untreated microbiota that normally inhibits *C. difficile* growth and toxin production also interferes with the bacterium QS activity, interference that could be abolished in a dysbiotic microbiota.
- Since probiotics support the intestinal microbiota by contributing to its intrinsic inhibition capacity, *C. difficile* virulence and QS activity would be limited in a dysbiotic microbiota that is also supplemented in probiotics.

The hypotheses formulated above led to the following objectives:

- To develop an *ex vivo* model in order to study various microbiota states; normal, antibiotic-disrupted and probiotic-supplemented.
- To characterize and compare the impact of normal and disrupted microbiota on *C. difficile agr* QS system modulation and toxin genes expression.

Finally, the means to achieve the objectives were:

- The CDI *in vivo* model of Chen *et al.* (2008) which uses a combination of antibiotics to induce susceptibility in mice was adapted and combined to *in vitro* co-cultures techniques in order to expose *C. difficile* cells to the microbiota and study its response in time. Viability was monitored by plating onto selective media and toxin production was measured by enzyme-linked immunosorbent assay (ELISA).
- RT-qPCR assays were designed through the creation and validation of sets of primers targeting *C. difficile* reference genes *(gluD, rpoA, rpsJ, adk)*, QS *agr* genes (*agrA2, agrC2, agrB1, agrD1)* and both toxin genes (*tcdA, tcdB*). Validation included the assessment of efficiency and specificity of the RT-qPCR assays as well as the assessment of reference genes stability.

Current mechanisms behind *C. difficile* establishment and pathogenesis into the host have not been fully elucidated yet. In addition, the overall knowledge about the *agr* QS and its impact in the regulation of virulence in *C. difficile* is limited. This study aimed to develop an *ex vivo* model in order to expose *C. difficile* cells to various live microbiota shaped following antibiotics and probiotics treatments. The combinaison of an *in vivo* model and culturomic and gene expression analysis techniques allowed to characterize the role of the intestinal microbiota and probiotic supplementation in modulation of *C. difficile agr* QS system. Investigating the impact of microbiota on *C. difficile* QS in a controlled and biologically relevant environment would provide understanding of the mechanisms potentially involved behind the colonization resistance of the microbiota.

2 MATERIAL AND METHODS

2.1 Bacterial strains and growth conditions

2.1.1 Clostridioides difficile

Toxigenic *C. difficile* strain R20291 was graciously provided by Professor Louis-Charles Fortier from University of Sherbrooke (QC, CAN) and stored at -80°C in brain heart infusion (BHI) broth (Fisher Scientific, MA, USA) supplemented with 20% (v/v) glycerol (Thermo Scientific, MA, USA). *C. difficile* was routinely cultured in Tryptose-Yeast Extract (TY) broth [30 mg/mL tryptose (Oxoid-Thermo Scientific, MA, USA), 20 mg/mL yeast extract (Fisher Scientific, MA, USA), pH = 7.4] and plated on Reinforced Clostridial medium (RCM) (Oxoid-Thermo Scientific, MA, USA) or Cycloserin Cefoxitin Fructose Agar (CCFA) (Anaerobe Systems, CA, USA). Cultures were incubated anaerobically in a controlled atmosphere of 5% H_2, 10% CO_2, and 85% N_2 at 37°C in an anaerobic workstation (Sheldon Manufacturing Inc., OR, USA). Prior to inoculation, all liquid and solid media were pre-reduced overnight in the anaerobic workstation.

2.1.2 Lyophilised probiotics strains

Lyophilized stock of probiotics strains *Lactobacillus acidophilus* CL1285®, *Lacticaseibacillus* (*Lactobacillus) casei* LBC80R® and *Lacticaseibacillus* (*Lactobacillus*) *rhamnosus* CLR2® was obtained from Bio-K+, a Kerry Company (QC, CAN) and stored at 4°C. Rehydration of lyophilized material was performed by adding 5.5 g of powder to 45.5 mL of warm sterile PBS (Sigma-Aldrich, MO, USA) and homogenized manually before mechanic homogenization in Stomacher 400 Circulator (Seward Ltd, SXW, UK) for 2 min. Bags were then laid on a flat surface for 30 min and mechanically homogenized a second time before serial dilution in sterile PBS. Enumeration of the probiotic suspension was performed by adding 1 mL of 10-fold serial dilutions to 30-35 mL of Man-Rogosa-Sharpe (MRS) agar (Sigma-Aldrich, MO, USA) at 45°C. Solidified culture in agar were then incubated between 48 hrs and 72 hrs under aerobic conditions at 37°C.

2.2 Growth of *C. difficile* in TY broth

Growth of *C. difficile* R20291 was performed in TY broth. First, cultures were produced by inoculating frozen aliquots of *C. difficile* in 25 mL of TY broth and allowed to grow for 15 hrs. The next day, 1 mL of the overnight culture was inoculated in 24 mL of fresh TY broth and allowed to grow overnight. The overnight culture was washed twice with 25 mL of sterile PBS then the cells were resuspended in 25 mL of fresh TY broth (mean OD = 2.6 ± 0.1). An inoculum of the cells suspension was added (1/100) to fresh TY browth and incubated at 37°C under anaerobic conditions. At various time points, a total of 3 mL samples were removed from culture to measure optical density (OD_{600}), pH and viable bacteria. Enumeration of viable cells was performed by 10-fold serial dilution in PBS and plated onto RCM media in technical triplicate. Plates were incubated at 37°C for 24 hrs under anaerobic conditions. Additional 1 mL samples were removed from culture and centrifugated at 4,500 ×*g* for 10 min. Filter-sterilization of the supernatant was performed using Filtropur S 0,2 uM filter (Sarstedt, QC, CAN) before being stored at 4°C.

2.3 Toxin quantification

Toxin production was measured in filtered cell-free supernatant that were stored at 4°C the day before. The concentration of toxins A and B were separately measured in the supernatant using the Separate detection of *C. difficile* toxins A and B ELISA kit (TGCbiomics, RP, GER) according to the manufacturer instructions. Depending on the samples, supernatants were diluted up to 1:10 in the dilution buffer provided prior analysis and toxin concentrations (ng/mL) were then adjusted to the dilution factor used. Color development was measured spectrophotometrically at an absorbance of 450 nm (ref 650 nm) in a Vmax microplate reader (Molecular Devices, CA, USA). The recombinant standard toxins included in the kit were used to generate calibration curves for toxin A and B. Linear portion of the curve was used to produce an equation of toxin concentration by volume of culture (ng/mL).

2.4 Animal model

2.4.1 Animals and housing

All animal experiments were approved by the Institutional committee for animal protection (CIPA) of the Institut National de la Recherche scientifique (INRS, QC, CAN) under protocol #1802-01. Seven-week-old healthy female C57/BL6 mice (n=84) were purchased from Charles River Laboratory (QC, CAN). Mice were housed in groups of 3 to 5 individuals for the same experimental group in plexiglass cages with autoclaved pine wood shaving bedding. Temperature (20°C), humidity (45%) and light conditions; cycle of 12 hours light-12 hours dark, remained constant for the duration of the experiment. Animal weights were measured everyday before other manipulations. Mice had *ad libidum* access to oval pelleted rodent feed and sterile drinking water unless stated otherwise. The mice were given a 7-day acclimation period after their arrival before any manipulation. Animals were divided in four experimental groups: (1) control (CTRL), (2) antibiotics only (ATB), (3) probiotics only (PRO), and (4) combination of antibiotics and probiotics (COMBIN). Mice from control group had access to normal rodent feed and sterile drinking water for the entire duration of the experiment. Mice from treatment groups (2, 3 and 4) were administered antibiotics only, probiotics only or a combination of both treatments as described below.

2.4.2 Antibiotic administration

Antibiotics were homogenized in sterile drinking water as described by Chen *et al.* (2008). Animals from groups ATB and COMBIN had *ad libitum* access to this solution for 3 days. The antibiotic cocktail included kanamycin (0.4 mg/mL), gentamicin (0.035 mg/mL), colistin (850 U/mL), metronidazole (0.215 mg/mL), and vancomycin (0.045 mg/mL). Those antibiotics were purchased from Sigma (MO, USA). New batch of antibiotics water was made each day in order to allow maximum stability of the compounds. After addition of the antibiotics, the supplemented water was filter-sterilized through a 500 mL 0.2 µM filter unit (Fisher Scientific, ON, CAN). After the intial antibiotic treatment of 3 days, antibiotic-water was switched to regular autoclaved water for 2 days. One day before euthanasia, mice received a single dose of USP grade clindamycin (Sandoz, QC, CAN) by intraperitoneal injection (10 mg/kg). Mice from CTRL and PRO groups were mock injected with USP grade 0.9% (NaCl) saline for injection (Baxter, ON, CAN). Those two groups received autoclaved water for the entire duration of the experiment and did not received antibiotics.

2.4.3 Probiotic administration

Animals from groups PRO and COMBIN received the equivalent of 1 billion CFU of probiotic by gastric gavage once a day for the duration of the treatment. Probiotic supplementation was prepared as follows. A mixture of *L. casei* LBC80R®, *L. rhamnosus* CLR2® and *L. acidophilus* CL1285® was prepared each day by homogenizing the lyophilized powder in sterile room temperature PBS following by vigorous shaking by hand until completed homogenization and an incubation for 30 min at room temperature before the gavage. Bacterial count in preparations were controlled by selecting few preparations randomly and by doing serial dilution and plating as previously described. The rigid metal gavage syringe was desinfected with isopropanol and rinsed with fresh PBS between each experimental group. Mice from CTRL and ATB groups were mock gavaged with fresh sterile PBS and did not received probiotics.

2.4.4 Organs and intestinal content collection for *ex vivo* model

The day following clindamycin injection, mice were euthanized by CO_2 asphyxia followed by cervical dislocation. All following procedures were done under sterile conditions in a biological safety cabinet. Abdomen was initially disinfected with isopropanol and opened longitudinally. The cecum-colon block was separated from other organs and conjonctive tissues and washed with sterile PBS. When the organs were completely isolated, cuts at the end of the ileum and the beginning of anus were made and the block cecum-colon was rapidly removed and immediately placed in a sterile container for transport of anaerobic tissus (Anaerobe System, CA, USA). Containers were then introduced into an anaerobic cabinet and organs were washed with sterile reduced PBS. Intestinal contents, including mucus and feces, were collected by pressing and pushing the content out of the GIT in a pre-weighted sterile 50 mL Falcon tube (Thermo Scientific). Intestinal contents from a total of 3 mice from the same experimental group were pooled and weighted. From this stage, these contents were considered as "intestinal microbiota" and were used as is for the *ex vivo* study. Since this study included three independent *ex vivo* experiments, a distinct and fresh pool of mice intestinal contents per experimental group was prepared for each individual experiment.

2.4.5 Organs collection for population study

A total of 12 additional mice per group were added for a gut microbial population study. Those mice received the same treatments as described previously with minor modifications. The day following the intraperitoneal injection, those mice were anesthetized by USP isoflurane (Fresenius Kabi, HE, GER). Abdominal cavity was opened, and the entire GIT was sampled and

chilled on ice. Following organs collection, the vena cava was cut, and mice were euthanized by exanguination. Each GIT section of interest (i.e., duodenum, jejunum, ileum, cecum and distal colon) were divided and luminal content and mucus were sampled separately. For content collection, sections were flushed with 1 mL of sterile PBS and directly poured in a sterile microcentrifuge tube (Fisher Scientific, MA, USA) except for cecum and distal colon (i.e., feces). The contents that were semi-liquid to solid were directly transferred in a tube. For mucus collection, the flushed sections were open longitudinally and mucus was scraped gently off the tissue and put in a sterile microcentrifuge tube. Content, mucus and feces samples were immediately snap frozen in liquid nitrogen then placed on dry ice. Frozen samples were stored at -80°C for long term storage.

2.5 Ex vivo model

2.5.1 Preparation of mid-log phase *C. difficile* cells

A starting culture of *C. difficile* was prepared as described earlier (see 2.2 Growth of *C. difficile* in TY broth). An inoculum of the suspension was added (1/100) to fresh TY broth and incubated at 37°C under anaerobic conditions for 7 hrs, reaching mid-exponential growth phase (DO_{600} = 1.45 ± 0.05).

2.5.2 Exposition of mid-exponential growth phase *C. difficile* cells to gut microbiota

A volume of 10 mL of mid-exponential phase *C. difficile* culture equivalent to 8.50 ± 0.03 Log_{10} CFU/mL were added per gram of fresh intestinal content pooled from 3 mice of the same experimental group (10:1 v/w). Co-cultures were then allowed to grow under anaerobic conditions at 37°C. A culture of *C. difficile* alone in TY was used as a reference culture. Samples were sampled after 1, 3 and 5 hrs of exposure as described below.

2.5.3 Cell-free supernatant collection

Cell-free supernatant samples for toxin quantification were obtained by collecting 1.5 to 2.0 mL of reference culture or co-culture and centrifuged at 4500 ×*g* for 10 min. Supernatants were filtered using Filtropur S 0.2 uM serynge filter (Sarstedt) before being stored at 4°C for a maximum of 24 hrs before toxin quantification using a commercial ELISA kit.

2.5.4 Cell collection and treatment for RNA extraction

Samples of 2 mL of reference culture or co-culture were added to 2 mL of ice-cold 100% ethanol-acetone (1:1) solution and homogenized. Ethanol and acetone were purchased from Les Alcools du Commerce (ON, CAN) and Thermo Scientific respectively. Treated and fixed cells were then immediately stored at -80°C for a maximum of 2 months until RNA extraction.

2.5.5 Cell collection for DNA extraction

Samples of 1 to 2 mL of co-culture were transferred in sterile microcentrifuge tubes and immediately stored at -80 °C for a maximum of 3 months until total DNA extraction.

2.5.6 *C. difficile* growth

Enumeration of viable *C. difficile* cells was performed by 10-fold serial dilution of samples from culture or co-cultures of *C. difficile* and gut microbiota. At each time point (i.e., 1, 3 and 5 hrs), sub-samples were withdrawn from the culture and co-cultures while maintaining the anaerobic growth conditions and serially diluted in PBS. Then, 100 µL of representative dilutions were plated onto CCFA plates in technical triplicate. CCFA media is specific and selective for *C. difficile* isolation as it contains sufficient amount of antibiotics cycloserin and cefoxitin which inhibits most other fecal bacteria. Plates were then incubated at 37°C for up to 24 hours under anaerobic conditions.

2.6 Molecular Biology methods

2.6.1 Total RNA extraction

Total RNA extraction was carried out using the RNeasy Mini kit (Qiagen, GER). Minor modifications in the manufacturer's protocol were applied in order to optimize cells lysis because of residual traces of mucus and other non-digestible products that was not successfully discarded with the washes and that could interfere with bacterial lysis. Modifications included the utilisation of a larger lysis buffer volume, rounds of six to seven 30 s of mechanical lysis in a FastPrep-24 sample preparation system and an additional washing step with RPE buffer. First, ethanol-acetone-treated cells were thawed on ice. Cells were then washed with 2 vol of ice-cold sterile PBS and collected by centrifugation at 5,100 ×*g* for 10 min at 4°C. Washed cells were resuspended in 1.5

vol of buffer RLT supplemented with 1% β-mercaptoethanol (Sigma-Aldrich, ON, CAN) and the mixture was distributed in 1.5 mL PCR-PT microtubes (Sarstedt) containing 0.5 mL of acid-washed 50-70 US sieve glass beads (Sigma-Aldrich). Cells were homogeneized using the FastPrep-24 sample preparation system (MP Biomedicals, CA, USA) at 4 m/s in intervals of 30 sec for a total of 180 sec. Between rounds, tubes were stored on ice to cool down for 5 min to prevent degradation due to overheating. After homogeneization, tubes were centrifugated at 1,000 ×*g* for 10 min to pellet cells debris. Supernatant containing RNA was then transferred to a nuclease-free 15 mL tube (FroggaBio, ON, CAN), making sure to avoid the beads and other solids at the bottom of the tube. One vol of 70% ethanol was added to the lysate and the content was pipetted up and down to mix carefully. Of this lysate, up to 700 µL was transferred to a RNeasy Mini spin column placed in a 2 mL collection tube and centrifuged at 10,000 ×*g* for 30 seconds. Flowthrough was discarded and the collection tube was reused for the remaining lysate and each column was reused for a maximum of three times. Because working with highly complex samples (i.e., high bacterial loads and trace of remaining mucus), 3 columns per sample were used to avoid clogging. Once all lysates were passed through the column, 700 µL of RW1 buffer was added and tubes were centrifuged at 10,000 ×*g* for 30 sec. Flowthrough and collection tubes were discarded and columns were placed in new collection tubes. A volume of 350 µL of RPE buffer was then added. Tubes were inverted few times manually to make sure that RPE buffer covered the entire walls and cap of the columns. Tubes were then incubated at room temperature for 1 minute before being centrifuged at 12,000 ×*g* for 30 sec. This washing step was repeated twice with additional 350 µL of RPE buffer. Third wash was carried out by centrifugating at 12,000 ×*g* for 2 min. Flowthrough was discarded after each centrifugation and collection tubes were reused. Following RPE buffer washes, columns were transferred in new collection tubes and centrifuged at maximum speed (25,000 ×*g*) for 1 min to dry the membranes and columns. Columns without any remaining liquid or drops were then placed in new collection tubes with cap and 50 µL of nuclease-free water was added in the center of the column's membrane. Tubes were centrifuged at 12,000 ×*g* for 1 min to elute RNA. To increase RNA concentration, eluate of the first column was reused to eluate the second column of RNA. RNA eluated from the 3 columns were pooled. RNA samples were kept on ice until a genomic DNA elimination was carried out or immediately stored at -80°C.

2.6.2 Elimination of genomic DNA from RNA samples

Contaminating genomic DNA was removed from RNA samples using the Turbo DNA-*free* Kit (Ambion, Austin, TX) following the rigourous DNA-free treatment according to manufacturer's instructions. Briefly, RNA was mixed with 0.1 vol of buffer and 1.5 µL of enzyme

and incubated for 30 min at 37°C. An additional 1.5 µL of enzyme was then added and the mix was incubated for a further 30 min at 37°C. A 0.2 vol of DNase Inactivation Reagent was then added to the previous mix and incubated for 5 min at room temperature before centrifugation at 10,000 ×*g* for 1.5 min. Supernatant containing clean RNA was collected and transferred to a new nuclease-free microcentrifuge tube (Ambion). At this point, 1.5 µL of sample was used to assess nucleic acids quantification and purity using 2000c Nanodrop spectrophotometer (Nanodrop Technologies Inc., DE, USA) and a 3.5 µL of sample was put aside for integrity assessement by gel electrophoresis. The remaining DNase-treated RNA was stored at -80°C until reverse transcription.

2.6.3 Agarose gel electrophoresis for quality assessment of RNA

All electrophoresis equipment and dishes were rinsed thoroughly with milliQ water and ELIMINase reagent (Decon Labs Inc., PA, USA) before utilization of RNA material. A 1% agarose gel was prepared by adding agarose (Fisher Scientific) to fresh 1X TAE buffer (Fisher Scientific) and heating the solution in a microwave for 2 mins using pulses of 30 sec until agarose was fully dissolved. After 3 min of cool down, 1.5 % of bleach 4% (La Parisienne, QC, CAN) was added to the solution and mix carefully. Gel was cast in an electrophoresis tray and set for at least 40 min before being submerged in fresh 1X TAE buffer in an electrophoresis tank (Thermo Scientific). Single strand RNA ladder (New England BioLabs, ON, CAN) prepared according to the manufacturer' instructions and negative control of nuclease-free water and loading dye buffer were included in the gel in single wells. RNA samples (3.5 µL) were treated with 2X RNA Loading dye (Thermo Scientific) according to manufacturer's instructions. A volume of 7 µL of samples were then loaded into individually wells. Gels were migrated at 90 V for 90 to 120 min until RNA bands were suitably separated. Following electrophoresis, tray was retrieved from the tank and put on a container containing a solution of 0.018% ethidium bromide (Thermo Scientific) in milliQ water. The gel was shaken on a Rocker system (Boekel Scientific, PA, USA) for 10 min and then rinsed with pure milliQ water for an additional 10 min. Gels were visualized under UV using a Gel Doc XR system (Bio-Rad, CA, USA) to assess integrity.

2.6.4 Reverse transcription (RT)

Reverse transcription was carried out using QuantiTect Reverse Transcription Kit (Qiagen, ON, CAN) as described by manufacturer's instructions. The first step was the genomic DNA elimination procedure. Reaction mix was composed of 2 µL of gDNA Wipeout buffer and 1 µg of template. If needed, total volume was brought to 14 µL with RNase-free water. Reactions were

incubated for 2 min at 42°C and then placed immediately on ice. RT mix was composed of 1 µL Quantiscript Reverse Transcriptase, 4 µL of Quantiscript RT buffer, 1 µL RT Primer mix and the entire genomic DNA elimination reaction (14 µL). For the no reverse-transcription control (NRT) reaction, the transcriptase enzyme was substituted with 1 µL of nuclease-free water. Reactions were incubated at 42°C for 15 min and then immediately at 95°C for 3 min. The cDNA samples were placed on ice before proceeding to real-time PCR (qPCR) or stored at -80°C until use.

2.6.5 Primer design

The primers used in this study were designed using online Primer3 software version 0.4.0 (Untergasser and Nijveen, 2007) and OligoQuest™ Tool (Integrated DNA Technologies, IA, USA). Parameters taken into consideration for primer design included, target annealing temperature of 60°C, minimal variability in forward and reverse primer's melting temperature, amplicon size ranging between 100 and 250 bp, primer size ranging between 18-24 bp and GC content ranging between 40% and 60%. Analysis of secondary structures formation such as hairpins, homo- and hetero-dimers was carried out using the OligoAnalyser Tool (Integrated DNA Technologies, IA, USA) according to their recommendations. Parameters taken into consideration for the formation of hairpins included the structure's ΔG (kcal.mole^{-1}) close to 0 or more positive and its Tm (°C) inferior to predicted reaction conditions. For the formation of primers dimers, primers that showed predicted secondary structures with a strong ΔG (-9 kcal/mol or more negative) were avoided. Selected primers were purchased from Integrated DNA Technologies. Primers were reconstitued in nuclease-free water to a concentration of 100 µM and further aliquoted at a concentration of 10 µM. Aliquots were stored at -20°C. Efficiency, specificity and reference genes' primers stability were assessed following the MIQE guidelines (Bustin et al., 2009). A total of 3 to 4 primers pairs for each gene were developed for validation purpose. Primer pairs demonstrating the best properties were used in the final assay. See Table 1 for selected primers pairs and optimized conditions.

2.6.6 Reaction efficiency

Reaction efficiency assessment was carried out by the construction of an 8-points standard curve using 2- or 5-fold dilution series of a reference template. Primer pairs demonstrating reaction efficiency between 90 and 110%, R-square superior to 0.98 and highly similar replicates quantification cycle (Cq) values were selected. If needed, primers concentrations and/or annealing temperature were adjusted and reaction efficiency was assessed again until satisfying previous

considerations. Optimized primers concentration and temperature were used for downstream analysis and final assays.

2.6.7 Assay specificity

Assay specificity was assessed by melting curve analysis and the predicted length of the fragments was verified by gel electrophoresis (see Agarose gel electrophoresis for PCR products visualization). Melting curve analysis was used to ensure that no nonspecific products or primers-dimers were formed. This was confirmed by the presence of a single strong peak in the melting curve analysis and a single band on gel. Moreover, target specificity for *C. difficile* genes was verified by using positive controls from DNA extracted from pure *C. difficile* culture and negative controls from DNA extracted from total gut microbiota without *C. difficile.* Cut-off for Cq intervals (ΔCq) between negative controls and positive controls was set to ≥ 4. Primers pairs that provide ΔCq inferior to 4 between negative and positive controls were rejected considering their poor specificity for *C. difficile* genes.

2.6.8 Stability of reference genes

After selection of the best primer pairs and optimization of reaction conditions, stability of reference genes used for normalization was assessed using the Reference Gene Selection Tool of the CFX Maestro Software (Bio-Rad, CA, USA). Various representative samples were used in the stability study such as cDNA samples from either pure culture of *C. difficile* at various growth time points and co-culture of *C. difficile* and microbiota at various exposition time points. Selection of the reference genes was made based on the software recommendations.

2.6.9 Real-time polymerase chain reaction (qPCR)

A total of 40 ng of cDNA were used in a 10 µL qPCR reaction volume using 2x SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) and 0.2 to 0.3 ρmol of forward and reverse primers. Hard-shell 96-wells PCR plates (Bio-Rad) were loaded with sample in technical triplicate and no reverse transcription control (NRT) and no template control (NTC) in technical duplicate. Real-time PCR was carried out using a CFX96 Thermocycler (Bio-Rad). The CFX Maestro Software was used to run the following conditions: DNA polymerase activation for 5 min at 95°C; 40 cycles comprising denaturation step of 15 sec at 95°C, annealing step of 30 sec at 60°C or 55°C and extension step of 30 sec at 68°C. Melting curve analysis was carried out from 50°C to 95°C with temperature increments of 0.5°C per 5 sec. A representative sample of the study as been

used as inter-run calibrator (IRC). IRC have been loaded in technical triplicate on all plates to normalize inter-run variation between targets assayed in separate qPCR runs.

2.6.10 Agarose gel electrophoresis for PCR products visualization

A 2% agarose gel was made by adding agarose powder to fresh 1X TAE buffer and heating in a microwave for 2 min (30 sec pulses and mixings) until agarose was fully dissolved followed by a cool down for 3 min. GelRed 10,000X (Biotium, CA, USA) was added to the gel (0.010%) and mixed carefully. Gel was then cast in an electrophoresis tray and set for at least 40 min before being submerged in fresh 1X TAE buffer in an electrophoresis tank. A 100 pb Plus DNA ladder (Thermo Scientific) and negative control of nuclease-free water and loading dye buffer were included in the gel. PCR product samples were retrieved from PCR plates, then mixed 5:1 with gel loading dye buffer and loaded (6 µL/wells) into individually wells. Gels were migrated at 90 V for 90 to 120 min until fragments bands were suitably separated. Following electrophoresis, gels were visualized under UV using a Gel Doc XR system (Bio-Rad).

2.6.11 DNA extraction

DNA extraction was carried out using the PowerSoil Pro DNA extraction kit (Qiagen). *C. difficile* and microbiota co-culture samples that were stored at -80°C were thawed on ice before being centrifuged at 10,000 ×*g* for 1 min to pellet solid matter. Supernatants were discarded and the pellets were weighted. Before proceeding with the manufacturer's instructions, samples were pre-treated as follow. A volume of 800 µL of CD1 solution was added to the pellet and homogenized. Samples were placed in a heating bloc set to 65°C for 10 min before being transferred to a second heating block set to 95°C for an additional 10 min. After the heat treatment, tubes were placed on ice for 10 min. Heat-treated samples were then used for DNA extraction following the kit manufacturer's instructions. DNA were eluated with 100 µL of C6 solution and placed immediately on ice. At this stage, a volume of 1.5 µL was used to assess quantification and purity using 2000c Nanodrop spectrophotometer and 5 µL of sample was put aside for integrity assessement by gel electrophoresis. The remaining DNA was stored at -80°C.

2.6.12 Agarose gel electrophoresis for integrity assessment of DNA

Agarose gel was prepared as described previously (see 2.6.11 Agarose gel electrophoresis for PCR products visualization). A 1 kB Plus GelPilot DNA ladder (Thermo Scientific) and negative control of nuclease-free water and loading dye buffer were included in the gel. DNA

samples were mixed 5:1 with gel loading dye buffer and loaded (6 µL/wells) into individually wells. Gels were electrophoresed at 100 V for 60 minutes until bands were suitably resolved. Following electrophoresis, gels were visualized under UV using a Gel Doc XR system (Bio-Rad, CA, USA).

2.7 Data and Statistical analysis

Normalized gene expressions were calculated using the ΔΔCq method included in the Bio-Rad CFX Maestro software and according to the formulas below. Relative changes and standard deviation of the mean (SEM) data were collected from Bio-Rad CFX Maestro software and were included in Excel sheet and reorganized. Target genes expression was normalized on the expression of the reference genes *rpoA* and *adk* (see Equation 1). Data are presented as fold-change expression relative to either the reference culture or the CTRL group depending on the analysis (see Equations 2 and 3), and therefore those have been set to 1. Fold-change data presented are mean of relative gene expression of biological replicates from three independent experiments. Data were then analyzed using Prism 9 (GraphPad software CA, USA). Statistical analysis using the Student T-test or the one-way ANOVA with the posthoc Tukey multiple comparaison test were performed for comparaison of expression levels between two groups or between more than two groups respectively. Means between groups (REF, CTRL, PRO, ATB, COMBIN) were compared within each exposure time point. Statistical significance lower or equal to 0.05 (95% confidence interval) was selected.

Equation 1 – Normalized expression

$$Normalized\ Expression_{Sample\ (GOI)} = \frac{RQ_{\ Sample\ (GOI)}}{\sqrt{\left(RQ_{\ Sample\ (Ref\ 1)} \times\ RQ_{\ Sample\ (Ref\ 2)}\right)}}$$

Where RQ is the relative quantity of a sample
Ref is the reference gene (the run includes two reference genes in each sample)
GOI = gene of interest (target)

Equation 2 – Fold Change [1]

$$Fold\ Change = \frac{Expression\ (experimental)}{Expression\ (control)}$$

Equation 3 – Fold Change [2]

$$Fold\ Change = -1/\left(\frac{Expression\ (experimental)}{Expression\ (control)}\right)$$

[1] Applicable if expression (experimental) > expression (control)
[2] Applicable if expression (experimental) < expression (control)

Table 1. Primers used in this study

Gene	Locus	Description	Primer	Sequence (5'-3')	Pro. size (pb)	Ann. Temp. (°C)
rpoA	CDR20291_0096	RNA polymerase subunit A	rpoA_MB1-F	GCTCTATCACAGGTGCAGATATAA	142	60
			rpoA_MB1-R	TCAGCAGAAACATAACCTCTACC		
rpsJ	CDR20291_0066	30S ribosomal protein S10	rpsJ_MB2-F	AGGACCTGTGCCTCTACCAA	132	60
			rpsJ_MB2-R	GGTGTTGGATTAGCTATGTCGAT		
adk	CDR20291_0088	Adenylate kinase	adk_MB1-F	CAGGTGCTGGTAAAGGTACTC	114	60
			adk_MB1-R	TTCCAAGTTCTGTTCCCTCTTT		
tcdA	CDR20291_0584	Toxin A	tcdA_MB1-F	ACTAGACGAACATGACCCATTAC	96	60
			tcdA_MB1-R	GCTACCGTTGCAGCTATAGATAA		
tcdB	CDR20291_0180	Toxin B	tcdB_MB1-F	AGCAGGAATTTCAGCAGGTAT	117	60
			tcdB_MB1-R	GCTCCTTCAGACTCAGCTAATG		
agrA2	CDR20291_3189	DNA-binding response regulator, lyttr family	agrA_MB3-F	TGGTATGGATACTGCTAGGAAGA	98	55
			agrA_MB3-R	CCTCATAACCTTCTTGCATAAACTC		
agrC2	CDR20291_3188	Sensor histidine kinase virs	agrC_MB3-F	TCCACTTAGTTGGAAAGAGGAAA	229	60
			agrC_MB3-R	CCTTCAGCAATCATTAATCCTAGCC		
agrB1	CDR20291_2640	Accessory gene regulator	agrB_MB2-F	GGAGGGTATCATGCAGACAATTA	151	55
			agrB_MB2-R	CACAAATTCCAACCCAACTAACA		
agrD1	CDR20291_2639	Autoinducer prepeptide	agrD_MB2-F	TGCTAGCTCATTGGCACTT	98	55
			agrD_MB2-R	GATTGCTGATTTCTTTGGGTACTT		

F: forward, R: reverse, Pro.: product, Ann.: Annealing, Temp.: Temparature, Ref.: reference.

3 DEVELOPPEMENT OF A QPCR ASSAY FOR GENE EXPRESSION ANALYSIS OF *AGR* QS SYSTEM IN *C. DIFFICILE*

3.1 Introduction

Real-time quantitative PCR (RT-qPCR) is considered a gold-standard method to quantitate changes in gene expression in response to specific environnemental conditions or specific treatments (Rocha, Castro, Aguiar, & Pacheco, 2020). In this study, RT-qPCR was used to compare changes in gene expression of four *C. difficile agr* QS genes; *agrA, agrC, agrB* and *agrD,* as well as both toxin genes; *tcdA* and *tcdB,* in response to exposure to various intestinal microbiota. Because of the high heterogenecity of co-culture samples that contained *C. difficile* but also intestinal indegenious bacteria, it was imperative to develop primers that would be highly specific to the target organism: *C. difficile* R20291. Reliability of gene expression measurements rests on the quality of extracted RNA and primers and the adequate selection of reference genes. The primers validation steps and the assessment of reference genes stability are presented in this chapter.

3.2 Results

3.2.1 Primer design

A total of four reference genes; *rpoA, rpsJ, adk* and *gluD,* and six genes of interest; *tcdA, tcdB, agrA2, agrC2, agrB1* and *agrD1,* were included in the study. For each of these gene, 3 to 4 primers pair candidates were designed and optimized. When designing the primers, key recommendations were considered such as melting temperature, a small amplicon size (≤ 250 pb), primer size and GC content (refer to Material and methods). Also, attention was put in preventing secondary structures such as hairpins and dimers, known for their negative effects on PCR efficiency. Primers were created using the protein coding sequence for each genes of interest of *C. difficile* R20291. Selection pipeline of the best candidates is described in Chapter 2. After determining the optimal temperature of each primer pairs with a temperature gradient assay, caracteristics such as specificity, efficiency and stability, especially for reference genes, were tested.

3.2.2 Specificity

Because of the high complexicity of gut microbiota, only primer pairs that demonstrated the highest specificity to *C. difficile* R20291 gene sequence were used in this study in order to prevent any non-specific amplification products. Specificity was tested by using cDNA of pure culture of *C. difficile* R20291 as positive control of amplification and DNA extracted from a pool of intestinal microbiota without *C. difficile* as negative control of amplification. Criteria for exclusion included a negative control Cq under 30 cycles and a Cq interval between the negative and the positive control smaller than 4 ($\Delta Cq < 4$). Cq interval was calculated by substracting Cq value of positive control to the Cq value of negative control ($\Delta Cq = Cq_{neg} - Cq_{pos}$). Also, the same Cq interval threshold was applied for exclusion of sample due to amplification in the no reverse transcription controls (NRT). Primer pairs that demonstrated the highest Cq interval and that did not meet any exclusion criteria were used for further RT-qPCR experiments (Table 2). The selected primer pairs showed Cq interval higher than 7.01 and amplification in the negative control all resulted in Cq value higher than 31.70, for these reasons, those primer pairs were assumed to be highly specific to *C. difficile* R20291. Amplification in the positive control resulted in Cq values ranging between 18.37 and 27.90.

Table 2. Primer pair specificity.

Primer pair	Mean Cq values		Cq interval	Conclusion
	Microbiota only	Positive control		
rpoA_MB1	36.93	22.98	13.95	Specific
rpsJ_MB2	31.70	23.20	8.50	Specific
adk_MB1	32.25	25.24	7.01	Specific
gluD_MB2	36.42	20.42	16.00	Specific
agrA2_MB3	-	27.54	NA	Specific
agrC2_MB3	-	27.90	NA	Specific
agrB1_MB2	38.40	26.07	12.33	Specific
agrD1_MB2	37.62	24.63	12.99	Specific
tcdA_MB1	38.85	18.37	20.48	Specific
tcdB_MB1	-	21.68	NA	Specific

"-": no amplification measured (Cq > 40); NA: no applicable (*in case where no amplification was measured in the negative control*).

3.2.3 Efficiency

Once the selected primer pairs showed great specificity for the target microorganism, the primers performances were evaluated with an efficiency assay using standard curves and the following criteria: Efficiency between 90% and 110%, Slope between -3.6 and -3.1 and R-square ≥ 0.995. The primer pairs that showed performance that met those criteria were used in further RT-qPCR experiment. Template used in those efficacy assays were cDNA or DNA extracted from pure culture of *C. difficile* R20291. Standard curves were constructed using 2-fold or 5-fold dilution series of the template. Efficiency assays have been conducted using the optimal temperature of each primers set determined previously (result not shown). Table 3 shows the standard curve data obtained by the efficiency assay of the selected primers sets used in this study and shows that all criteria have been met. In the case of agrC2_MB3, initial efficiency assay conducted with a primer concentration of 0.3 µM, leads to poor performance and an efficiency of 132.8% (data not shown). Efficiency assay was repeated with a lower primer concentration (i.e., 0.2 µM) which lead to better performance (Efficiency =101.1%) and the meeting of all criteria.

Table 3. Primer pair efficiency.

Primer pairs	Efficiency (%)	R-square	Slope	y-int	Template dilution	[Primers] (µM)	Temp (°C)
rpoA_ MB1	98.4	0.998	-3.362	21.688	5-fold	0.3	60
rpsJ_ MB2	96.4	0.997	-3.411	21.911	5-fold	0.3	60
adk_MB1	100.0	0.998	-3.322	22.389	5-fold	0.3	60
gluD_MB2	91.8	0.999	-3.536	26.665	5-fold	0.3	60
agrA2_ MB3	104.0	0.999	-3.230	28.738	2-fold	0.3	55
agrC2_ MB3	101.1	0.998	-3.295	30.344	2-fold	0.2	60
agrB1_ MB2	102.8	0.996	-3.257	24.805	2-fold	0.3	55
agrD1_ MB2	106.9	0.999	-3.167	23.626	2-fold	0.3	55
tcdA_ MB1	107.1	1.000	-3.163	26.484	2-fold	0.3	60
tcdB_ MB1	102.7	0.999	-3.259	29.883	2-fold	0.3	60

Int: Intersection; [Primers]: primers concentration; Temp: temperature.

3.2.4 Stability of the reference genes

In order for a reference gene to be used for expression normalization, they have to demonstrate great stability across a variety of sample types. The stability assay was carried out using the Reference gene selection tool integrated in the Maestro CFX software. For the selection of those genes, stability assay was carried out using four representative sample types (i.e., 4 conditions): pure *C. difficile* culture in TY after 2 and 6 hrs and co-cultures of *C. difficile* and intestinal microbiota after 2 and 6 hrs. Average M value was automatically calculated by the software and converted in stability value [Ln(1/AvgM)]. Since the M-value and gene stability are negatively correlated, the most stable genes exibit the lowest M-value. The software then classed the genes based on their stability value and included them in one the following categories: Ideal, Acceptable and Unstable. Ideal reference genes are stable and represent minimal variations across the tested samples while unstable reference genes represent excessive variation across tested samples. It is recommended to exclude the latter from analyses and to use any ideal reference gene for normalization. The median category (i.e., Acceptable) included reference genes that are not ideally stable and represent moderate variation across the samples tested. While the use of ideal reference genes is strongly recommended over any acceptable genes, in the case where no ideal gene is present, one can use three or more acceptable reference genes. Table 4 shows stability of the four primer sets for reference genes *rpoA, rpsJ, adk* and *gluD* in those 4 conditions. The stability analysis shows that only *gluD* was unstable in the conditions tested which resulted in its exclusion. Since the other three reference genes; *rpoA, rpsJ* and *adk,* have been evaluated as ideal reference genes and the normalization against a single reference gene is generally not acceptable according to the MIQE guidelines (Bustin et al., 2009), all three genes have been included in the further gene expression study.

Table 4. Assessment of reference genes stability.

Order	Gene	Primers ID	Evaluation	Avg M-Value	Stability (Ln(1/AvgM))	# Conditions
1	*rpsJ*	rpsJ_MB2	Ideal	0.185	1.691	4
2	*rpoA*	rpoA_MB1	Ideal	0.184	1.691	4
3	*adk*	adk_MB1	Ideal	0.303	1.193	4
4	*gluD*	gluD_MB1	Unstable	1.689	-0.524	4

ID: identification; Avg: average.

3.2.5 Primer validation

Primer sets were validated after each step throughout the optimisation assay to ensure the amplicons produced showed the expected size on agarose gel. Figures 5-7 show representative gel profiles of the selected primer sets PCR amplicons on 2% agarose gel stained with 0.01% GelRed. First of all, Figures 5-7 show that PCR products obtained from PCR runs in various positive samples are the expected sizes. Secondly, Figures 5-7 show that only a single band is obtained for each amplification which demonstrates that a single specific product is amplified. Those results are confimed by the presence of a single and strong peak for each primer set in the melting curve analysis (Figures 8-10). The melting curve analysis also confirms the absence of any primer-dimer formations.

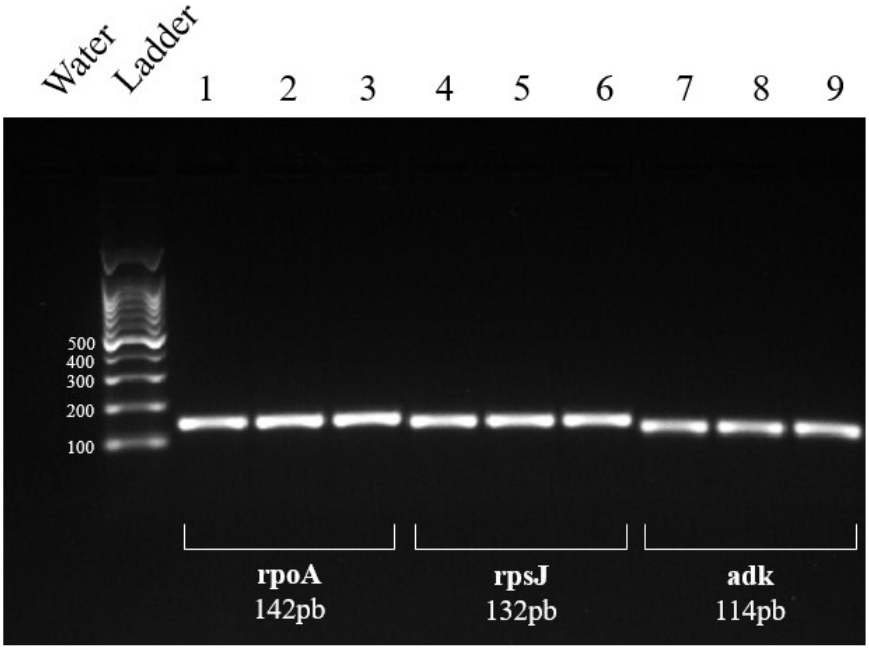

Figure 5. Representative gel electrophoresis image of reference genes *rpoA, rpsJ* and *adk* amplification products by PCR.

Ladder: 100 pb molecular weight marker. Lanes 1, 2 and 3: *rpoA,* Lanes 4, 5 and 6: *rpsJ,* Lanes 7, 8 and 9: *adk.* Molecular weight of the five lowest bands of the marker have been added on the figure. Expected molecular weight of the amplicons have been added on the figure.

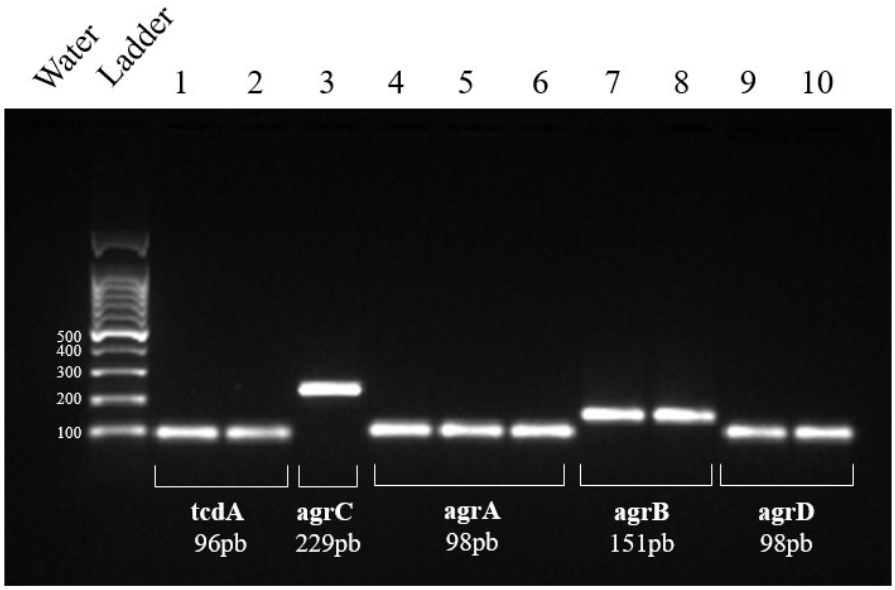

Figure 6. Representative gel electrophoresis image of target genes *tcdA, agrC, agrA, agrB* and *agrD* amplification product by PCR.

Ladder: 100 pb molecular weight marker. Lanes 1 and 2: *tcdA,* Lane 3: *agrC,* Lanes 4, 5 and 6: *agrA,* Lanes 7 and 8: *agrB,* Lanes 9 and 10: *agrD.* Molecular weight of the five lowest bands of the marker have been added on the figure. Expected molecular weight of the amplicons have been added on the figure.

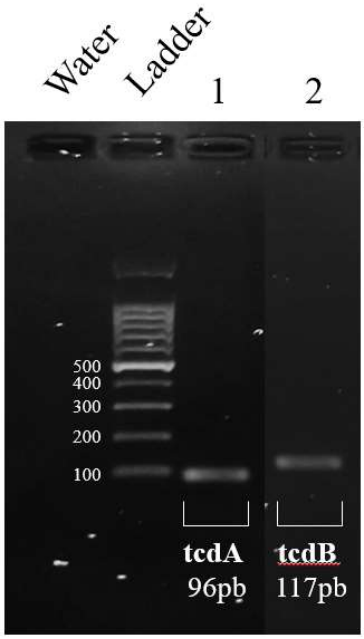

Figure 7. Representative gel electrophoresis image of target genes *tcdA* and *tcdB* amplification product by PCR.

Ladder: 100 pb molecular weight marker. Lanes 1: *tcdA,* Lane 2: *tcdB.* Molecular weight of the five lowest bands of the marker have been added on the figure. Expected molecular weight of the amplicons have been added on the figure.

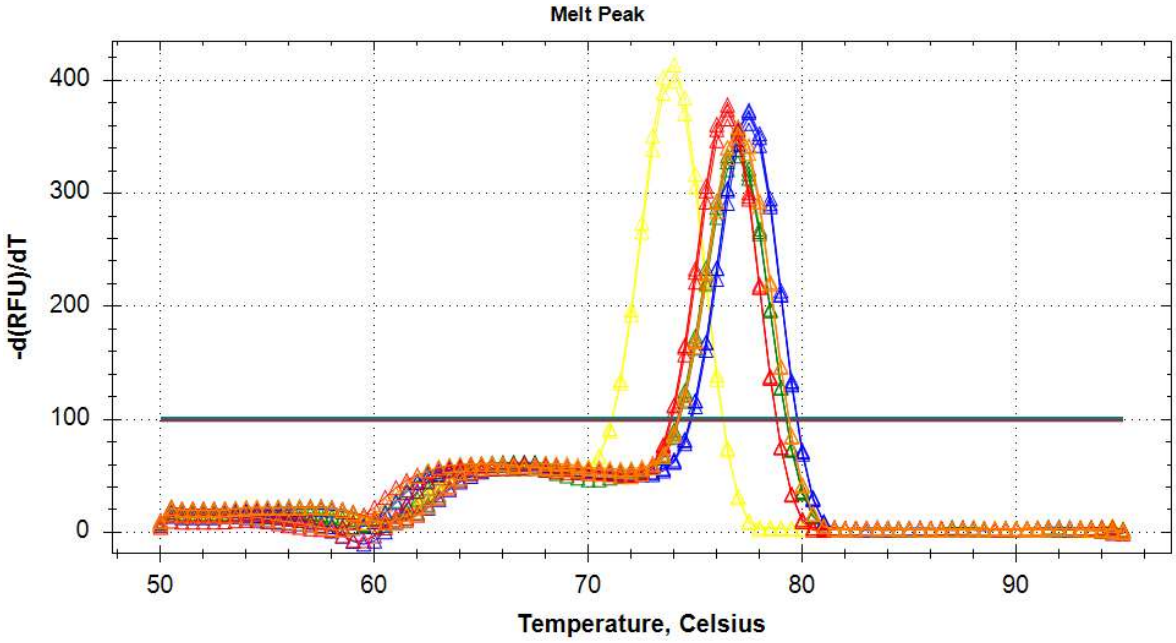

Figure 8. Representative RT-qPCR melting curve analysis of *rpoA* (red), *rpsJ* (blue), *adk* (green), *tcdA* (orange) and *agrC* (yellow).

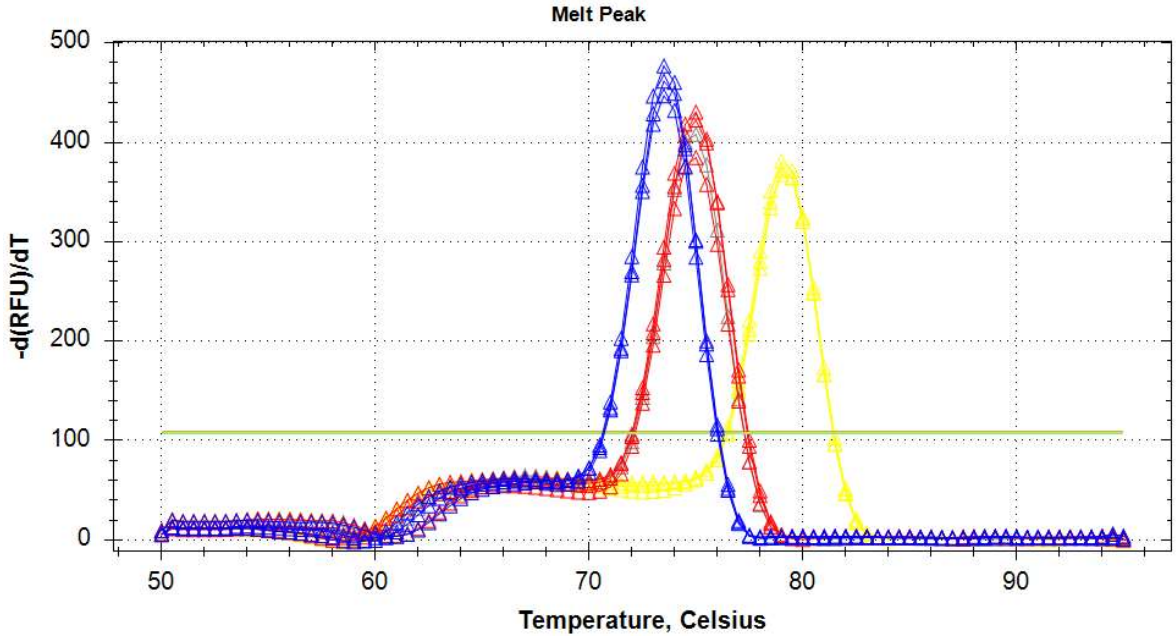

Figure 9. Representative RT-qPCR melting curve analysis of *agrA* (red), *agrB* (blue) and *agrD* (yellow).

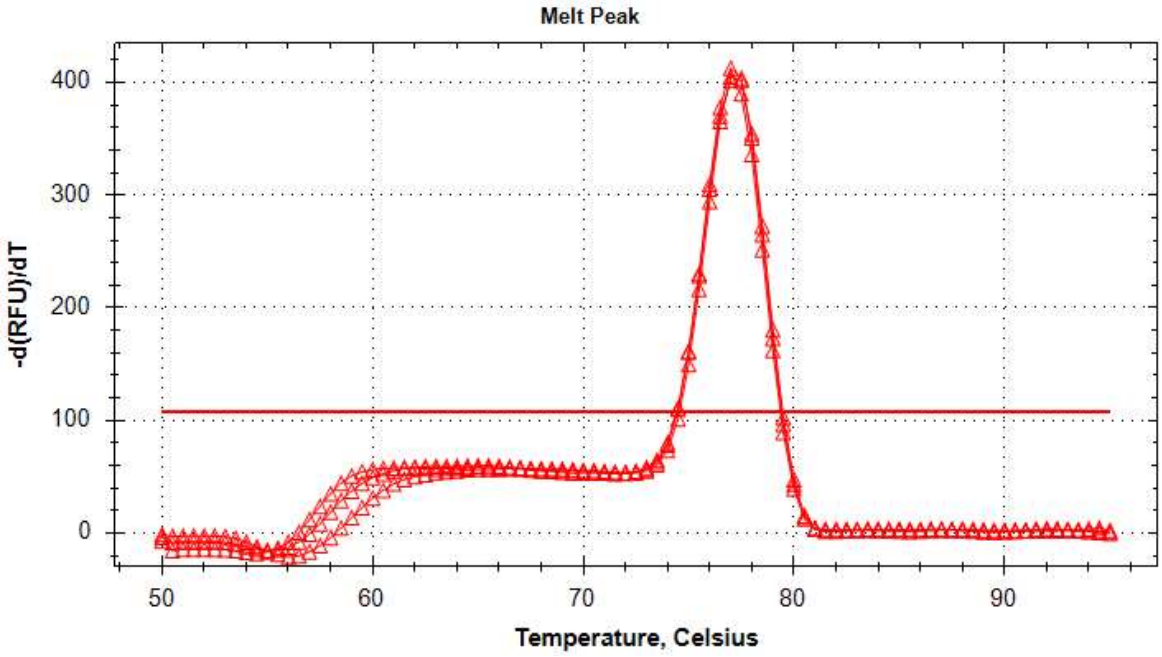

Figure 10. Representative RT-qPCR melting curve analysis of *tcdB*.

4 INTESTINAL MICROBIOTA EXPOSURE MODULATES *AGR* QUORUM SENSING SYSTEM IN *C. DIFFICILE*

4.1 Introduction

4.1.1 Current models to study the virulence of *C. difficile*

Study models for the investigation of *C. difficile* establishment and cytotocixity are essential in order to understand the pathogen behaviour and patterns of infection in humans and mammals. Infections and diseases comprise the invasion of the pathogen and its establishment into a specific niche, but also the host immune system responses to the infectious agent as well as the pathogen response itself to the host defense mechanisms. More than often, those highly nutrient-rich metabolic environments, inter-cellular responses and biochemical prosesses cannot be replicated in laboratory settings with standard growth media and non-dynamic culture techniques, hence the use of animal models to capture the inherent complexity of the mammal's physiology. Animal models in the study of *C. difficile* virulence and pathogenesis have included hamsters, mice, rabbits and guinea pigs (Best et al., 2012). These animals are used in *C. difficile* infection studies because they show great similarities in their susceptibility to CDI after antimicrobial treatment that is also found in humans (Chen et al., 2008). It is worth to mention that the association between *C. difficile* toxin and antibiotic-associated colitis was established nearly three decades ago with the use of the hamster model (Best et al., 2012). While animal models represent the complexity of the infection settings, the study of molecular and cellular mechanisms in addition to the bacterial interactions between resident and pathogenic bacteria are limited in those models.

Few *in vitro* and batch culture fermentation models to study the impact of intestinal microbiota on *C. difficile* have emerged in the last decades (Borriello & Barclay, 1986; Borriello et al., 1988; Gaisawat, Iskandar, MacPherson, Tompkins, & Kubow, 2019; Gaisawat, MacPherson, et al., 2019; Pultz & Donskey, 2005). Most of them use fecal preparation commonly called fecal water/slurry or faecal emulsion prepared by homogeneizing human or animal feces in water or PBS. Borriello & Barclay (1986) were pionners in that domain with the publication of their "*in vitro* model of colonisation resistance to *Clostridium difficile* infection" and were among the firsts to determine the capacity of the normal gut flora to prevent growth and toxicity of *C. difficile.* They discovered that viability and toxin production of *C. difficile* were inhibited when the pathogen was grown in faecal emulsions, but those inhibitory effects were lost when the faecal emulsions were

sterilised, highlighting the importance of viable bacteria (Borriello & Barclay, 1986). Other more complex systems have later been developed such as the three-stage compound continuous culture system and the triple-stage chemostat gut model to mimic physiological and ecological environment of the gut (Crowther et al., 2014; Macfarlane, Macfarlane, & Gibson, 1998). Those systems allow for the control of various parameters like the pH, nutrient concentrations, gaseous environment and other physicochemical and microbiological characteristics (Drake, 2002). Despite the fact that those models enable to study complex interactions between microbial populations under various nutritional and microbiological states as well as allowing longitudinal investigations, continuous culture methods require intensive efforts, are expensive and represent high risk of waste in case of contamination.

4.1.1 *Quorum sensing* systems are usually not studied in those models

The key role of QS systems in the modulation of virulence in pathogenic bacteria has been unequivocally established (Deep et al., 2011; Elvers & Park, 2002; Hammer & Bassler, 2003; Ohtani et al., 2002). However, there is a lack of interest in those signalling systems in terms of their implication in colonization resistance mechanisms against pathogenic intruders and in particular, *C. difficile.* Only a handful of studies have examined the impact of QS systems *agr* and Lux_{CD} on the pathogen virulence and even less have investigated impact of QS depleted variants during *in vivo* infection models. Nonetheless, interest is rising in targeting QS systems as potential therapeutic strategies with the use of QS quenching (QQ) molecules and inhibitors (QSIs) (de Kievit & Iglewski, 2000; Gray, Hall, & Gresham, 2013; Gunaratnam, Diarra, et al., 2021; Remy et al., 2018; Rutherford & Bassler, 2012). There is a possibility that intestinal microbiota possess an intrinsic capacity to inhibit or interfere with the QS of invading pathogen by direct or indirect mechanisms, preventing their establishment into the host or expression of virulence factors. To our knowledge, no one have previously investigated *C. difficile'*s QS system modulation by the intestinal microbiota as part as of a potential mechanism of colonisation resistance.

4.1.2 Seeking a more controlled and biologically relevant model

In order to study cellular mechanisms such as the QS systems and the effects of a normal and altered intestinal microbiota on those systems, there was a need for a model that would offer increased control of the environment, but at the same time, needed to be more biologically relevant than growth media alone. Thus, an *ex vivo* model was developed in the purpose of exposing *C. difficile* cells to various viable intestinal microbiota from mice treated with antibiotics and/or

probiotics. The combinaison of an *in vivo* and culturomic techniques allowed for a greater control on bacterial ratios and exposition time. Microbiota profiles included in this study were normal microbiota (CTRL), normal microbiota supplemented with probiotics (PRO), antibiotic-disrupted microbiota (ATB) and antibiotic-disrupted microbiota supplemented with probiotics (COMBIN). In order to produce the microbiota, an *in vivo* model of CDI has been adapted. In this model, a combination of antibiotics namely, kanamycin, colistin, metronidazole, vancomycin, gentamycin and clindamycin has been used to induce susceptibility in mice (Chen et al., 2008). In this present study, subgroups of non-treated and antibiotic-treated mice have been supplemented in probiotics daily. At the end of the treatments, mice were euthanazied and ceacal and colonic contents were sampled and added to mid-exponential phase *C. difficile* cells. Viability, toxin production and QS and toxin gene expression analysis have been performed and compared between groups.

4.2 Results

4.2.1 Growth of *Clostridioides difficile* R20291 in TY

In order to select the growth time of *C. difficile* that will be exposed to microbiota, growth of R20291 isolate was monitored. Optical density (OD_{600nm}) was measured in three independent *C. difficile* cultures over a period of 11 hrs (Figure 11). In our laboratory conditions, R20291 isolate reaches exponential phase after 4 hrs of growth and reaches late-exponential phase after 9-10 hours of growth. According to the growth curves, stationnary phase was expected to be reached after 11 hrs. To benefit from the maximal *agr* QS system activity that was expected to be reached in mid- to late-exponential phase, when both cell density and viability could be optimal, the 7-hrs time point was selected to collect cells that were used in the *ex vivo* assay. In this model, 7-hrs bacteria were exposed to microbiota for 1 hr, 3 hrs and 5 hrs, leading to the onset of stationary phase at 12-hrs growth time.

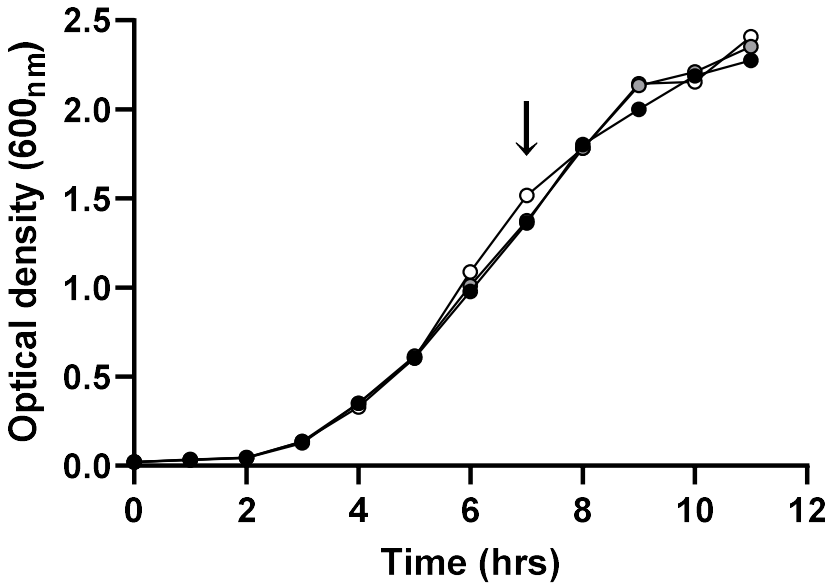

Figure 11. Growth of *C. difficile* R20291 in TY.

Washed cells of an overnight culture of *C. difficile* in TY were diluted 1:100 in fresh TY and grown during an 11 hrs period. Culture was sampled every hour. Results shown are OD_{600nm} curve of 3 biological replicates (n=3). Arrow indicates the time point selected for the preparation of test cells in the *ex vivo* assay.

4.2.2 Viability of *C. difficile* R20291 exposed to intestinal microbiota

In order to investigate if *C. difficile* viability would be affected by the presence of microbiota, viability was monitored at each exposure time points in co-cultures of *C. difficile* and 10-fold diluted microbiota isolated from either non-treated control mice (CTRL), probiotic-fed mice (PRO), antibiotic-treated mice (ATB) and mice that received combination of both probiotics and antibiotics (COMBIN). *C. difficile* was also grown in TY (REF) and used as a control. Total CFU/mL was enumerated by serial dilution in PBS and plated onto CCFA agar which contain antibiotics that inhibit most of the anaerobic microbiota. Sub-samples of co-cultures were collected after 1 hr, 3 hrs and 5 hrs following exposition to microbiota. Viables cells (Log_{10} CFU/mL) before exposure (0 hr) and at each time points after exposure were enumerated in biological replicates from three independant experiments (Figure 12). Data show that what would be the "final phase of expansion" - or the late exponential phase - of *C. difficile* seems limited in the presence of all samples containing microbiota tested after 3 and 5 hrs. After 5 hours in this model, viability of *C. difficile* exposed to microbiota was statistically significantly reduced when compared to the reference culture (REF) ($P \leq 0.05$). When microbiota were compared together, statistical analysis

showed no difference between the groups ($P > 0.05$). Otherwise, One-way ANOVA showed no significant difference between pH variations over time between groups ($P = 0.47$), with the strongest acidification that occurred in the reference culture (ΔpH T0-T5 = -0.25) (Table 5).

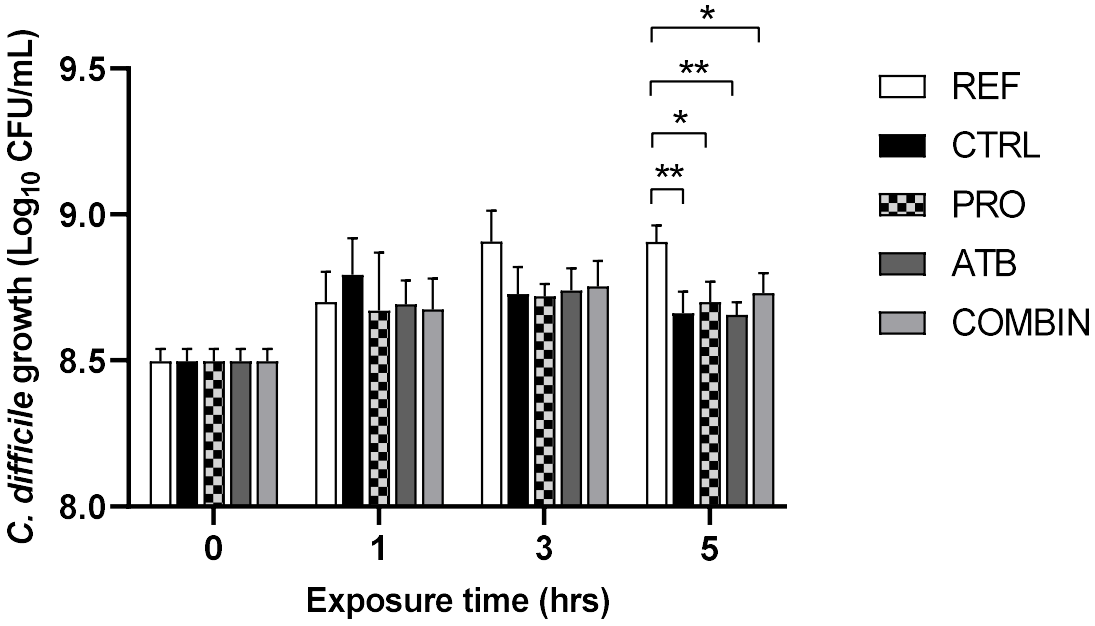

Figure 12. Growth expansion of *C. difficile* in TY is limited in presence of microbiota.

Mid-exponential phase *C. difficile* cells were exposed for 1, 3 or 5 hours to various 10-fold diluted microbiota isolated from control mice (CTRL), probiotic-fed mice (PRO), antibiotics-treated mice (ATB) and mice that received combination of both probiotics and antibiotics (COMBIN). Reference culture consisted of mid-exponential phase *C. difficile* cells grown in sterile TY media (REF). Results shown are the mean of biological replicates from 3 independant experiments (n=3). Error bars indicate standard errors of the means. One-way Anova with Tukey multiple comparaison test: * $P \leq 0.05$, ** $P \leq 0.01$.

Table 5. pH evolution over time in reference culture and co-culture of *C. difficile* and microbiota.

Culture		Initial pH	Final pH	pH variation	SEM	*P* #
Reference	**REF**	6.67	6.43	-0.25	0.096	
Co-cultures	**CTRL**	6.65	6.44	-0.21	0.009	0.47
	PRO	6.55	6.49	-0.06	0.075	
	ATB	6.56	6.46	-0.10	0.034	
	COMBIN	6.77	6.52	-0.24	0.145	

REF: reference, CTRL: control, PRO: probiotics, ATB: antibiotics, COMBIN: combination, SEM: standard errors of the means, ns: non significant. #One-way ANOVA analysis, confidence interval = 95%.

4.2.3 Toxin production by *C. difficile* R20291

To address whether the exposure of microbiota would induce virulence of *C. difficile* and further stimulate an early toxin production (*i.e.* before reaching stationary phase where toxin production is usually maximal), supernant sampled from the reference culture and co-culture at every time points were sterilized by filtration and toxin A and toxin B concentrations were measured using ELISA kit. This ELISA assay enables the separate quantitation of toxin A and toxin B as matter of ng of toxin per volume of suspension (mL) by using standard curve from dilution series of purified toxins supplied in the kit. Cell-free supernants were tested undiluted and diluted 2 to 10 times in the buffer supplied in the kit to ensure that absorbance measured was comprised in the linear portion of the standard curve. Toxin concentration was then adjusted with the dilution factor, if applied. Over the period of the experiment (5 hrs), toxin production increased in all co-culture and reference culture (Figure 13). After 1 h, levels of toxin A were similar in all groups and similar to levels measured in the reference culture (2.07 ng/mL) but difference become more noticable after 3 and 5 hrs. Surprisingly, compared to the toxin production in the reference culture (3.06 ng/mL at 3 hrs and 3.46 ng/mL at 5 hrs), normal microbiota slightly stimulated toxin production in CTRL mice group (3.44 ng/mL at 3 hrs and 4.48 ng/mL at 5 hrs) and PRO mice group (3.33 ng/mL at 3 hrs and 4.75 ng/mL at 5 hrs). On the opposite, toxin levels were generally lower when cells were exposed to antibiotics-disrupted microbiota: ATB (2.38 ng/mL at 3 hrs and 3.16 ng/mL at 5 hrs) and COMBIN (2.24 ng/mL at 3 hrs and 3.65 ng/mL at 5 hrs) groups. However, statistical analysis showed no significant difference between the groups ($P > 0.05$). Otherwise, Toxin B levels did not reached the assay detection threshold for all exposure time tested and the results could not be presented in this study.

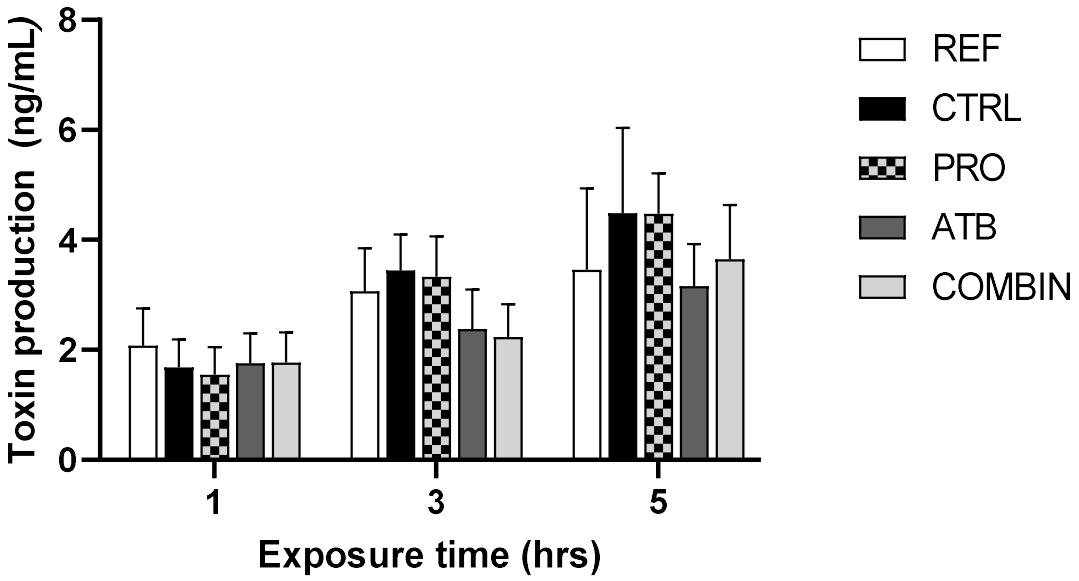

Figure 13. Production of toxin A by *C. difficile* in the presence of microbiota.

Mid-exponential phase *C. difficile* cells were exposed for 1, 3 or 5 hrs to 10-fold diluted microbiota isolated from control mice (CTRL), probiotic-fed mice (PRO), antibiotics-treated mice (ATB) or mice that received combination of both probiotics and antibiotics (COMBIN). Reference culture consisted of mid-exponential phase *C. difficile* cells growth in sterile TY media (REF). Toxin A concentration was measured in cell-free supernatant from co-culture by ELISA for separate detection of toxins A and B. Results shown are the mean of biological replicates from 3 independant experiments (n=3). Error bars indicate standard errors of the means. One-way Anova showed no significant difference between the five groups within each exposure time ($P > 0.05$).

4.2.4 RNA extraction and quality control

RNA was extracted from cells pellet from reference cultures of *C. difficile* R20291 (REF) and from co-cultures of *C. difficile* and intestinal microbiota (CTRL, PRO, ATB and COMBIN), at each exposure time points (T1, T3 and T5) and from three independent experiments (1, 2 and 3). The RNA extraction method used was the RNeasy Mini kit (Qiagen, UK) with minor modifications (see Material and Methods). Bioanalysis parameters of all RNA extraction are presented in Table 6 (See Chapter 6. Appendice). RNA integrity was analysed by running a 3 µL volume of DNAse-free treated RNA sample on a 1% agarose gel. Figures 14 and 15 show representative vizualisations of the RNA integrities from the five groups. RNA isolated from reference cultures showed acceptable integrity with strong and clear 16S (3000 bp) and 23S bands (1500 bp). However, RNA samples showed some level of degradation which is indicated by the ratio 23S:16S not being 2:1 and the presence of smears. As shown in Figure 14, RNA isolated from co-cultures with microbiota demonstrated increased degree of degradation from moderate (Figure 14 Lanes 7, 8 and 9) to important degradation (Figure 14, Lanes 4, 5 and 6). Generally, RNA isolated from co-cultures

with normal and healthy microbiota (i.e. CTRL and PRO) demonstrated higher degradation degrees than disrupted microbiota (i.e. ATB and COMBIN). While degraded RNA sample are not ideal and could potentially resulted in false gene expression results, all of the samples were included in the next step (i.e., cDNA synthesis and RT-qPCR). Amplification curves were then carefully analyzed to ensure homogeneity of Cq tendencies across samples from the same type from the 3 independent experiments and any suspicious amplification resulted in its exclusion of the gene expression analysis. At the end, only 1 replicate of probiotic group at 3 hrs (PRO 3 T3) was excluded from the analysis.

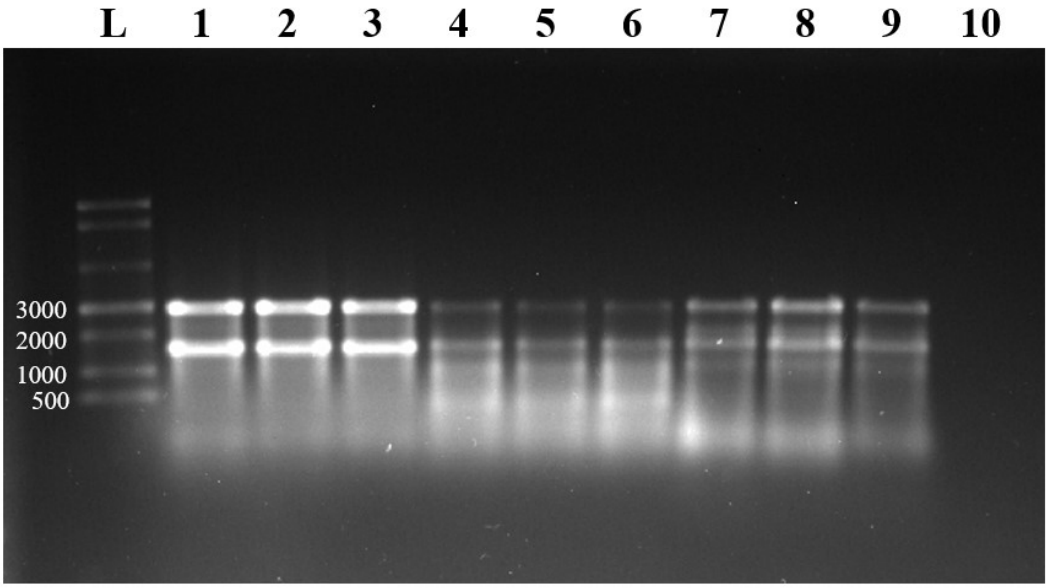

Figure 14. Representative gel electrophoresis image of extracted and DNase-treated RNA samples from reference culture and co-culture of *C. difficile* and microbiota.

L: ssRNA molecular weight marker. Lanes 1 to 3: reference cultures T1, T3 and T5, Lane 4 to 6: CTRL cultures T1, T3 and T5, Lanes 7 to 9: ATB cultures T1, T3 and T5, Lane 10: water with loading buffer. Molecular weight of the four lowest bands of the marker have been added on the figure. Expected rRNA 16S and rRNA 23S can be identified by the 3000 bp and 1500 bp bands.

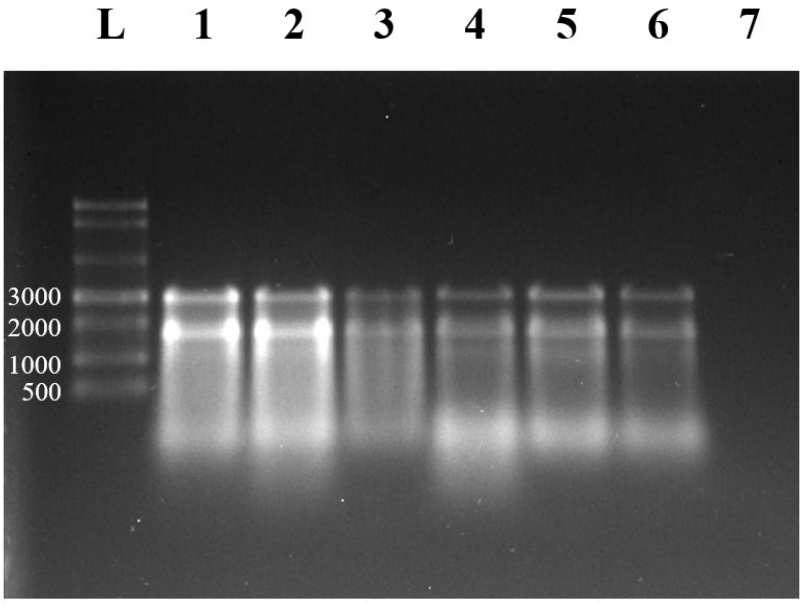

Figure 15. Representative gel electrophoresis image of extracted and DNase-treated RNA samples from co-cultures of *C. difficile* and microbiota.

L: ssRNA molecular weight marker. Lanes 1 to 3: PRO cultures T1, T3 and T5, Lane 4 to 6: COMBIN T1, T3 and T5, Lanes 7: water with loading buffer. Molecular weight of the four lowest bands of the marker have been added on the figure. Expected rRNA 16S and rRNA 23S can be identified by the 3000 bp and 1500 bp bands.

4.2.5 Comparative analysis of QS and toxin gene expression of strain R20291 exposed to microbiota.

First, there was an interest in investigating how *C. difficile* QS system would be influenced by the microbiota. In order to determine if the presence of microbiota could have an effect on QS and toxin gene expression, we analyzed by quantitative RT-PCR the relative expression of QS genes *agrA* and *agrC* of the second Agr locus and *agrB* and *agrD* of the first Agr locus as well as toxins genes *tcdA* and *tcdB* in *C. difficile* R20291 exposed to normal intestinal microbiota (CTRL) at different exposure times (1, 3 and 5 hrs) compared to cells grown in sterile TY (REF). This section presents the gene expression analysis between normal microbiota to the gene expression measured in the reference culture (REF) of *C. difficile* grown in standard growth conditions with no addition of microbiota. Gene expression in the REF group have been set to 1. Gene expression has been normalized on *rpoA* and *adk* RGs, which demonstrated ideal stability accros all samples.

Results show that expression of all QS genes were up-regulated after 3 and 5 hrs compared to the expression measured in the reference culture (Figure 16). T-test showed no significant difference between the two groups ($P > 0.05$) regardeless of the exposure. Interestingly, the up-regulation measured in *agrB* and *agrD* were of higher amplitude than relative expression of *agrAC* yet, high variability in gene expression did not leads to significant difference between groups.

tcdA and *tcdB* were up-regulated after 1 h in CTRL group with increase of 2.20-fold and 1.68-fold respectively (Figure 17) while both genes were down-regulated after longer exposure times (3 and 5 hrs) with little to no change between time points. However, statistical analysis showed no significant difference in *tcdA* or *tcdB* expression between the two groups ($P > 0.05$). It appeared that normal microbiota had a rapid effect of stimulating expression of toxins gene, but that effect was then lost after a further incubation time.

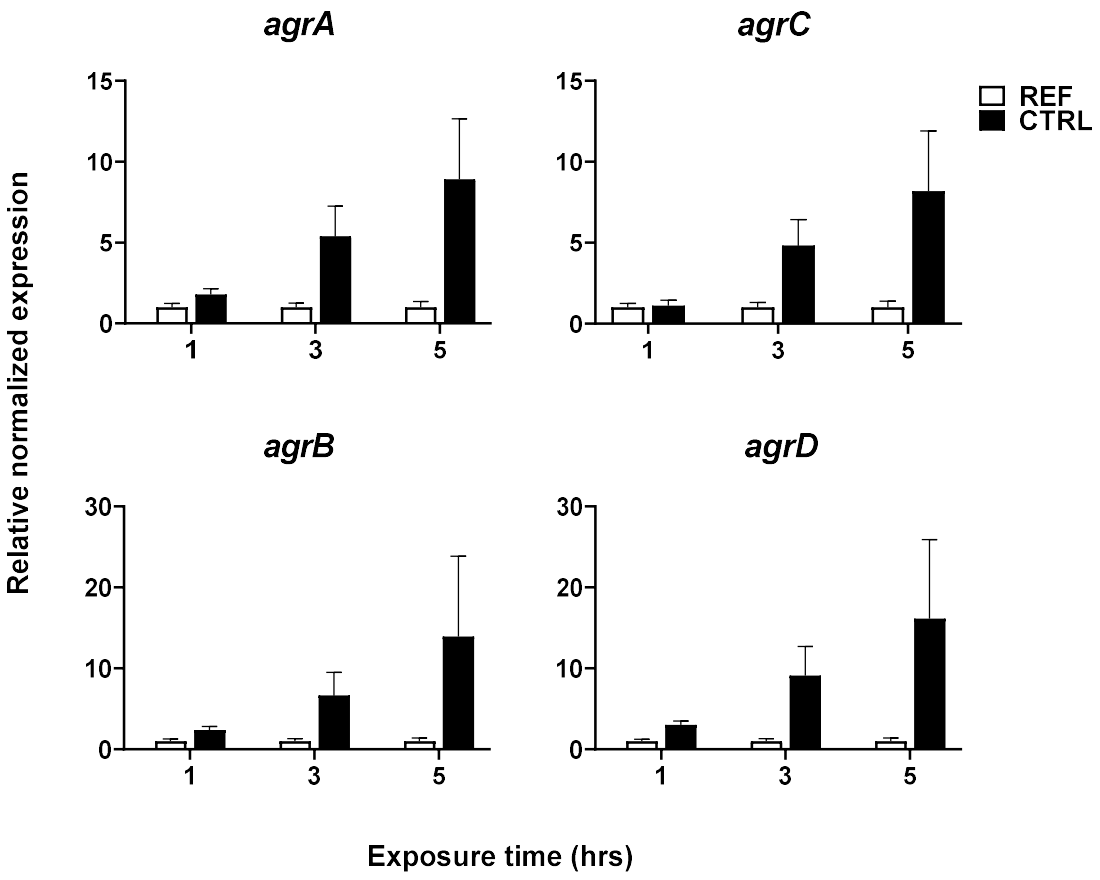

Figure 16. Relative normalized expression of *agrA, agrC, agrB* and *agrD* in *C. difficile* exposed to microbiota compared to reference culture.

Mid-exponential phase *C. difficile* exposed for 1, 3 or 5 hours to 10-fold diluted microbiota isolated from control mice (CTRL). Reference culture consisted of mid-exponential phase *C. difficile* cells growth in sterile TY media (REF). Results shown are the mean of biological replicates from 3 independant experiments (n=3). Expressions were normalized on reference genes *rpoA* and *adk.* Expressions of REF group are set to 1. Expressions of CTRL group are relative to REF group expression. Error bars indicate standard errors of the means. T-test within each exposure time ($P > 0.05$).

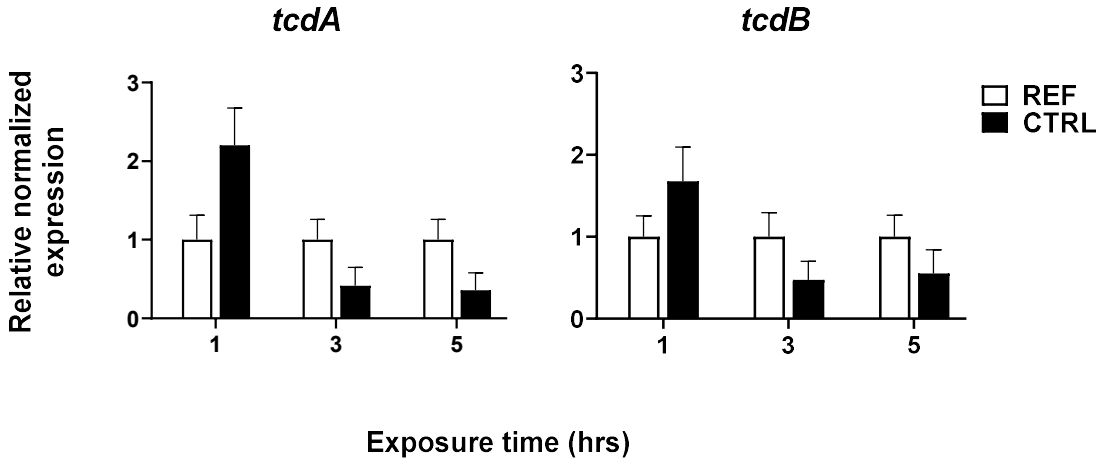

Figure 17. Relative normalized expression of *tcdA* and *tcdB* in *C. difficile* exposed to microbiota compared to reference culture.

Mid-exponential phase *C. difficile* exposed for 1, 3 or 5 hours to 10-fold diluted microbiota isolated from control mice (CTRL). Reference culture consisted of mid-exponential phase *C. difficile* cells growth in sterile TY media (REF). Results shown are mean of 3 independant experiments (n=3). Expressions were normalized on reference genes *rpoA* and *adk.* Expressions of REF group are set to 1. Expressions of CTRL group are relative to REF group expression. Error bars indicate standard errors of the means. T-test within each exposure time ($P > 0.05$).

4.2.1 Comparative analysis of QS and toxin gene expression of strain R20291 grown in normal and antibiotic-altered microbiota.

Our understanding of the potential implication of the intestinal microbiota in regulating the QS of intruder, and in particular *C. difficile,* is almost inexistant. First and foremost, a better appreciation of how QS system is regulated in normal and disrupted microbiota environment is needed. In this section, the effects of a normal microbiota supplemented in probiotics (PRO), a disrupted microbiota following antibiotics consumption (ATB) and a disrupted microbiota supplemented in probiotics (COMBIN) on QS genes, *i.e. agrACBD* and *tcdA* and *tcdB* expression were compared to the effect of a normal microbiota (CTRL).

4.2.1.1 agrA

In *C. difficile* R20291*, agrA* gene can be found on the second Agr locus (*agr*2) and encodes for a response regulator of the LytTR family. While the role of AgrA has been elucidated in *S. aureus,* its role in *C. difficile* is still unclear, but there is evidence of the potential regulation role of AgrA in virulence of *C. difficile* (M. J. Martin et al., 2013). Our analysis showed that expression of *agrA* only differs significantly between ATB and COMBIN group after 3 hrs (Figure 18). In fact, compared to the CTRL group, *agrA* expression was up- and down-regulated by 2.0-fold in the

respective groups, but those difference were not significant ($P > 0.05$). Nonetheless, one-way ANOVA analysis showed that *agrA* expression relative to control was significantly lower in COMBIN group compared to ATB group ($P \leq 0.05$). Surprisingly, this difference in expression was lost after 5 hrs exposure. Otherwise, fold change in *agrA* did not showed significant difference between the groups after 1 and 5 hrs ($P > 0.05$).

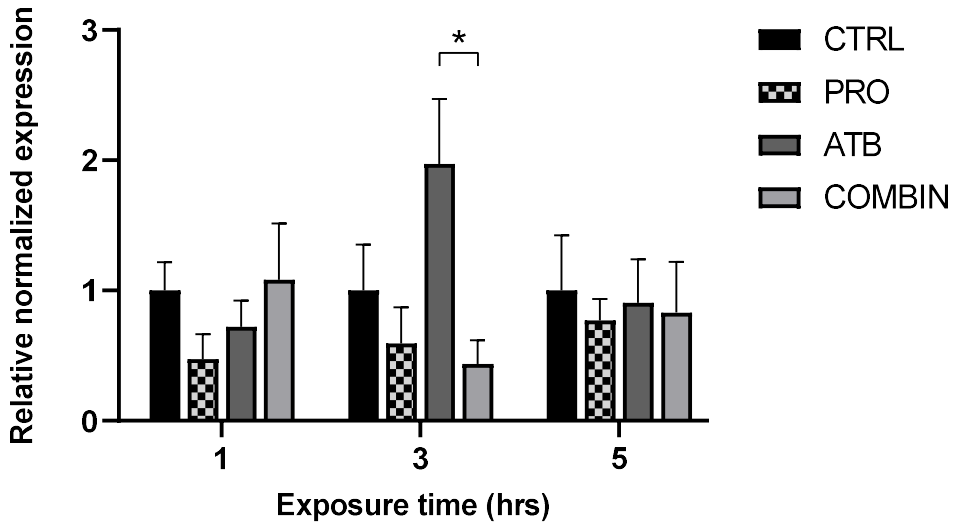

Figure 18. Relative normalized expression of *agrA* in *C. difficile* exposed to microbiota.

Mid-exponential phase *C. difficile* exposed for 1, 3 or 5 hours to 10-fold diluted microbiota isolated from control mice (CTRL), probiotic-fed mice (PRO), antibiotics-treated mice (ATB) or mice that received combination of both probiotics and antibiotics (COMBIN). Results shown are the mean of biological replicates from 3 independant experiments (n=3). Expressions were normalized on reference genes *rpoA* and *adk.* Expressions of CTRL group are set to 1. Expressions of treatment groups are relative to CTRL group expression. Error bars indicate standard errors of the means. One-way Anova with Tukey multiple comparaison test: * $P \leq 0.05$.

4.2.1.2 agrC

agrC encodes for a signal transducer that is responsible to sense the auto-inducer peptide (AgrD) at the surface of the cell. Little is known about the regulatory role of *agrC* in the virulence of *C. difficile.* It is worth to mention that *agrA* and *agrC* are thought to form a single transcriptional unit because an insertion in *agrA* causes the underexpression of *agrC* (Melissa J. Martin et al., 2013). Data showed a relatively similar patterns for *agrC* relative expression then previously seen in *agrA* which could be expected if they form a single transcriptional unit (Figure 19). Compared to the CTRL group, *agrC* expression was up-regulated by 1.57-fold in the ATB group and down-regulated by 2.78-fold in the COMBIN group. Furthermore, relative expressions tended to be

decreased in both PRO and COMBIN groups by 1 and 3 hrs respectively. However, statistical analysis showed no significant difference in *agrC* expression between the groups ($P > 0.05$).

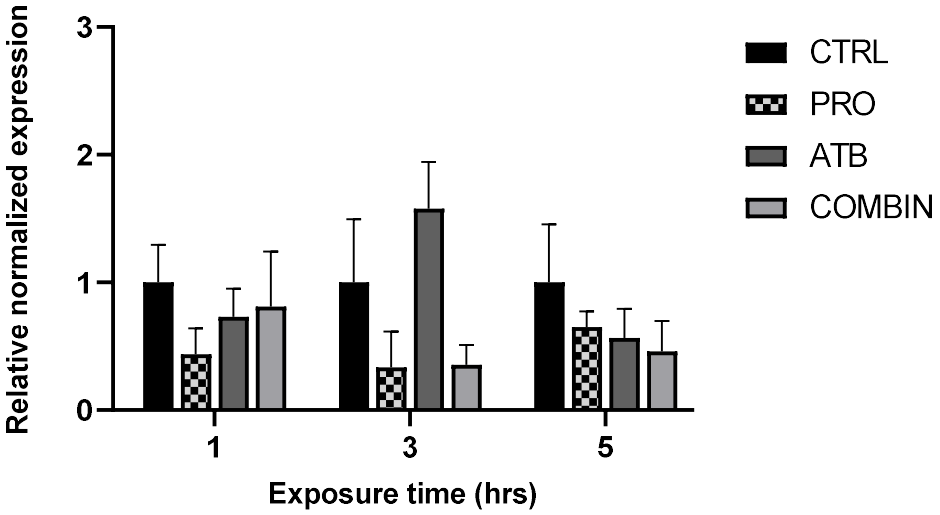

Figure 19. Relative normalized expression of *agrC* in *C. difficile* exposed to microbiota.

Mid-exponential phase *C. difficile* exposed for 1, 3 or 5 hours to 10-fold diluted microbiota isolated from control mice (CTRL), probiotic-fed mice (PRO), antibiotics-treated mice (ATB) or mice that received combination of both probiotics and antibiotics (COMBIN). Results shown are the mean of biological replicates from 3 independant experiments (n=3). Expressions were normalized on reference genes *rpoA* and *adk*. Expressions of CTRL group are set to 1. Expressions of treatment groups are relative to CTRL group expression. Error bars indicate standard errors of the means. One-way Anova with Tukey multiple comparaison test ($P > 0.05$).

4.2.1.3 agrB

In *C. difficile, agrB* gene can be found on both *agr* loci; *agr*1 and *agr*2. A study has shown the first locus, *agr1* is essential for *C. difficile* virulence whereas *agr2* was not (Darkoh et al., 2016). For this reason, only *agrB1* has been included in this study. AgrB is a transmembrane protein involved in the maturation and release of AIP (*i.e.* AgrD). *agrB* was strongly over-expressed in ATB group after 3 and 5 hrs compared to the CTRL group (Figure 20). Fold change of *agrB* in ATB group was significantly increased by 5.11-fold ($P \leq 0.05$) and 6.37-fold ($P \leq 0.01$) at 3 and 5 hrs exposure respectively. In the COMBIN group, *agrB* relative expression increased by 4.30-fold after 5 hrs in the COMBIN group. However, this increase was not significant ($P = 0.10$). Interestingly, as seen in *agrA* expression, expression of *agrB* also differs significantly between ATB and COMBIN groups at 3 hrs exposure ($P \leq 0.01$) but not after 5 hrs ($P = 0.43$). While not significant, *agrB* relative expression in the COMBIN group at 5 hrs was 2.07-fold lower than the

relative expression in the ATB group. Statistical analysis showed no significant difference in *agrB* expression between CTRL and PRO groups at neither time points ($P > 0.05$).

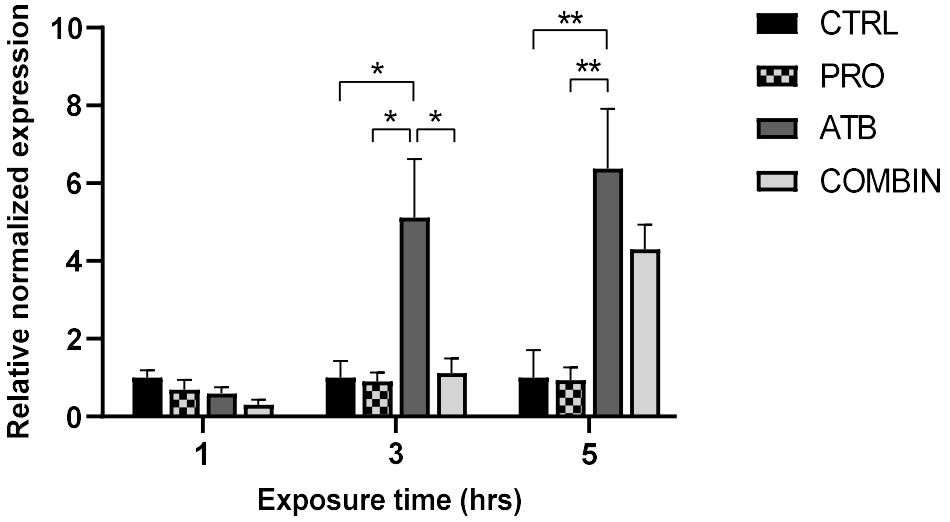

Figure 20. Relative normalized expression of *agrB* in *C. difficile* exposed to microbiota.

Mid-exponential phase *C. difficile* exposed for 1, 3 or 5 hours to 10-fold diluted microbiota isolated from control mice (CTRL), probiotic-fed mice (PRO), antibiotics-treated mice (ATB) or mice that received combination of both probiotics and antibiotics (COMBIN). Results shown are the mean of biological replicates from 3 independant experiments (n=3). Expressions were normalized on reference genes *rpoA* and *adk.* Expressions of CTRL group are set to 1. Expressions of treatment groups are relative to CTRL group expression. Error bars indicate standard errors of the means. One-way Anova with Tukey multiple comparaison test: * $P \leq 0.05$, ** $P \leq 0.01$.

4.2.1.4 agrD

As for *agrB, agrD* genes can be found on both *agr* loci. Because it has been demonstrated that the *agr1* locus is essential for *C. difficile* virulence (Darkoh et al., 2016), only *agrD1* has been included in this study. AgrD encodes for the precursor of a small cyclic peptide that acts as an extracellular signal in the *agr* QS system. Relative expression profiles of *agrD* is nearly identical to *agrB* (Figure 21). Data show once again a strong over-expression in ATB group after 3 and 5 hrs and in COMBIN group after 5 hrs compared to the CTRL group. Fold change of *agrD* in ATB group was significantly increased by 4.74-fold ($P \leq 0.05$) and 7.83-fold ($P \leq 0.01$) at 3 and 5 hrs exposure respectively. In the COMBIN group, *agrD* relative expression increased by 4.80-fold after 5 hrs in the COMBIN group. However, this increase was not significant ($P = 0.17$). As seen in *agrB* expression, relative expression differs significantly between ATB and COMBIN groups by 3.7-fold ($P \leq 0.01$) after 3 hrs. While not significant, relative expression between the two groups

differs again by 3.04-fold ($P > 0.05$) after 5 hrs. Statistical analysis showed no significant difference in *agrD* expression between CTRL and PRO groups at neither time points ($P > 0.05$).

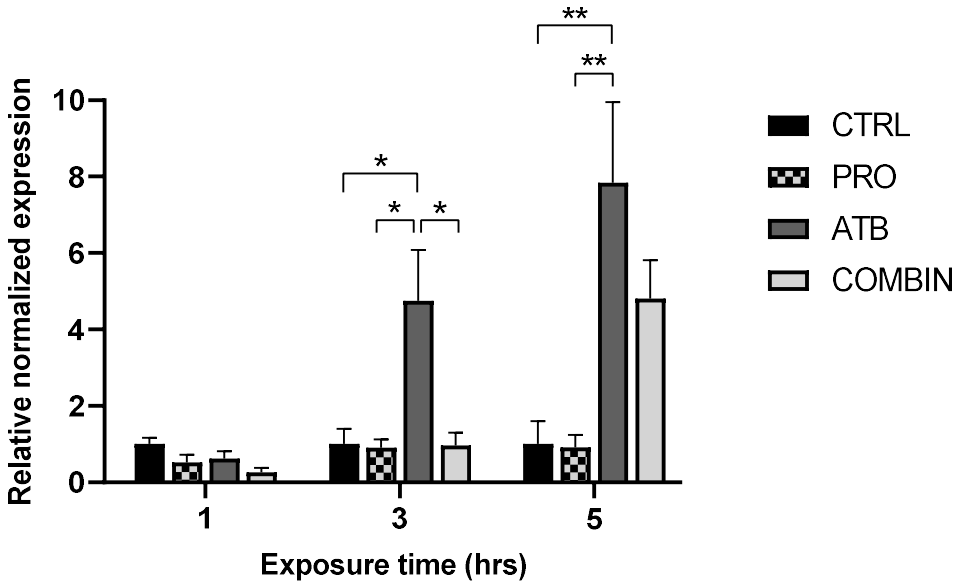

Figure 21. Relative normalized expression of *agrD* in *C. difficile* exposed to microbiota.

Mid-exponential phase *C. difficile* cells were exposed for 1, 3 and 5 hrs to 10-fold diluted microbiota isolated from control mice (CTRL), probiotic-fed mice (PRO), antibiotics-treated mice (ATB) or mice that received combination of both probiotics and antibiotics (COMBIN). Results shown are the mean of biological replicates from 3 independant experiments (n=3). Expressions were normalized on reference genes *rpoA* and *adk*. Expressions of CTRL group are set to 1. Expressions of treatment groups are relative to CTRL group expression. Error bars indicate standard errors of the means. One-way Anova with Tukey multiple comparaison test: * $P \leq 0.05$, ** $P \leq 0.01$.

4.2.1.5 *tcdA* and t*cdB*

Compared to CTRL group, *tcdA* and *tcdB* genes were generally under-expressed in all treatment groups at all time (Figure 22). Unexpectedly, both gene expressions were slightly up-regulated in the PRO group at 3 hrs but not after 5 hrs. *tcdA* and *tcdB* were down-regulated in ATB group (*tcdA:* -6.25-fold, *tcdB:* -3.57-fold) and even more in COMBIN group (*tcdA:* -12.5-fold, *tcdB:* -6.67-fold) compared to CTRL group after 1 h, resulting in a significant decrease of *tcdA* expression in COMBIN group ($P < 0.05$). While both relative gene expression in ATB group rapidly reach CTRL levels after 3 and 5 hrs, expressions in COMBIN group were still importantly down-regulated after 3 hrs. Despite the under-expression trends observed in ATB and COMBIN groups, statistical analysis generally showed no significant difference between the groups ($P > 0.05$).

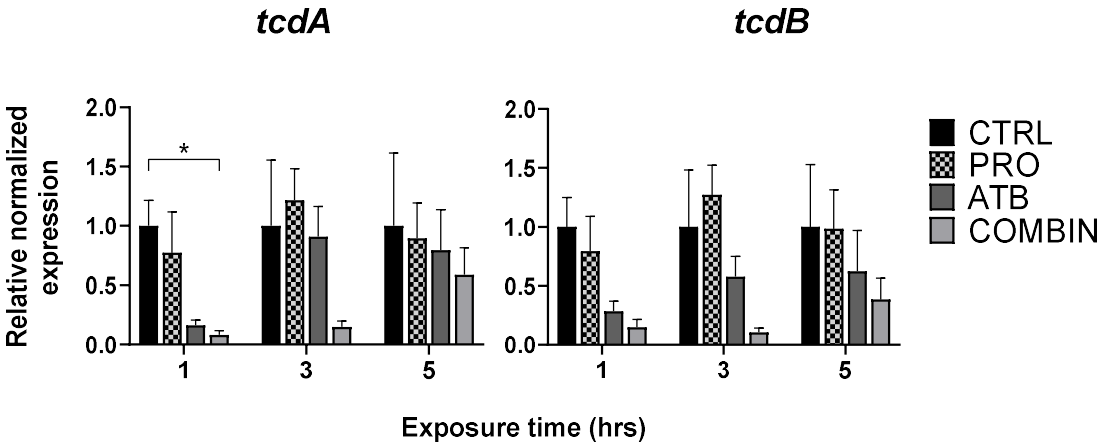

Figure 22. Relative normalized expression of *tcdA* and *tcdB* in *C. difficile* exposed to microbiota.

Mid-exponential phase *C. difficile* cells were exposed for 1 hour, 3 hours or 5 hours to 10-fold diluted microbiota isolated from control mice (CTRL), probiotic-fed mice (PRO), antibiotics-treated mice (ATB) or mice that received combination of both probiotics and antibiotics (COMBIN). Results shown are mean of 3 independant experiments (n=3). Expressions were normalized on reference genes *rpoA* and *adk*. Expressions of CTRL group are set to 1. Expressions of treatment groups are relative to CTRL group expression. Error bars indicate standard errors of the means. One-way Anova with Tukey multiple comparaison test: * $P \leq 0.05$.

5 DISCUSSION

5.1 Development and validation of a RT-qPCR assay for gene expression analysis of *agr* QS system in *C. difficile*

The Minimum Information for Publication of Quantitative Real-Time PCR Experiment (MIQE) guidelines were developed by Bustin *et al.* (2009) in order to increase integrity, consistency and transparency of RT-qPCR data acquisition and analysis in the scientific literature. Thus, to provide the most reliable results possible, the MIQE guidelines were followed when designing this RT-qPCR assay. The validation steps demonstrated that the selected primers offered specific and efficient product amplification. Specificity validation was carried out by demonstrating the absence of positive amplification in negative samples that did not contain the target organism, as wells as providing evidence of a single amplified product of the expected size on electrophoresis gel and a single peak in the melting profile. PCR efficiency was tested for all primer pairs in order to ensure good PCR performance. The results demonstrated that all efficiency profiles respected the selection criteria: efficiency between 90% and 110%, slope between -3.6 and -3.1 and R-square $\geq$ 0.995. Also, the dynamic linear range covered at least 6 orders of magnitude (data not shown). The reference gene stability assay demonstrated that 3 of 4 RGs were stable across the four representative samples tested. Thus, all three RGs; *rpoA, rpsJ* and *adk* have been selected for the normalization and were used in the *ex-vivo* experiment. This last assay was performed again in the final *ex vivo* samples to ensure adequate stability of the selected RGs. In the end, RG *rpsJ* showed acceptable stability when tested in the final samples while *rpoA* and *adk* showed ideal stability. Therefore, all target genes (i.e., *agrA, agrC, agrB, agrD, tcdA* and *tcdB*) expressions were normalized on both ideally stable genes.

5.2 *C. difficile* viability in the presence of intestinal microbiota

It was previously determined by others that healthy viable intestinal microbiota inhibits *C. difficile* growth *in vitro.* This was first demonstrated by Borriello *et al.* (1986) who inoculated *C. difficile* cells in faecal emulsions from stools of healthy adults. They showed that growth of *C. difficile* was inhibited when grown in these emulsions, but that inhibition was alleviated when the emulsions were sterilised, which was an indication that the inhibitory effect was attributable to the viable bacteria (i.e., microbiota). A a second study by Borriello *et al.* (1988) used that model to

predict the impact of antibiotics treatment on their potency to induce CDI susceptibility. Caecal emulsions made by suspending and diluting 20-fold w/vol the cecal content of hamsters in distilled water were used. They showed that when *C. difficile* was cultured in cecal content isolated from hamsters treated with certain antibiotics (clindamycin or ampicillin), growth of the bacterium was supported, and toxin production occurred following a 48 hrs incubation period. However, growth of *C. difficile* was inhibited in cecal content from hamster that received other antibiotics (aztreonam or temocillin) and from hamsters that was not treated. Those findings were supported by infecting the animals with *C. difficile.* Hamsters that received clindamycin or ampicillin died while the others did not develop a susceptibility to *C. difficile* and survived (Borriello et al., 1988). Their hamster model of colonization resistance to CDI was later adapted to test a wider range of antimicrobials for their capacity to induce susceptibility to CDI in mice (Donskey et al., 2015; Nerandzic & Donskey, 2011).

In this present study, we adapted and merged the *in vitro* model from Borriello *et al.* (1986) and a mouse *in vivo* CDI model developed by Chen *et al.* (2008). The latter used a cocktail of six antibiotics: kanamycin, colistin, metronidazole, vancomycin, gentamycin and clindamycin to induce susceptibility in mice (Chen et al., 2008). Instead of infecting the mice with *C. difficile* inoculum as it is done in the original *in vivo* model, the caecal and colonic contents were isolated from non-treated and antibiotics-treated mice and added to mid-exponential *C. difficile* culture. The resulting co-cultures were then incubated for a total of 5 hours. Based on the evidence stated above and because the total viable microbiota was used, we hypothesized that *C. difficile* viability would be negatively impacted in the presence of non-treated normal microbiota. Inversely, a microbiota isolated from mice treated with antibiotics would be permissive to *C. difficile* growth. The results showed that the presence of microbiota has indeed interfered with *C. difficile* growth expansion. However, in this model, *C. difficile* viability was found only midly affected by the presence of the microbiota. At the beginning of the experiment, a total of 8.50 Log_{10} CFU/mL were exposed to 10-times (w/vol) diluted fresh cecal and colonic content isolated from mice that received probiotic and antibiotic treatments: control mice (CTRL), probiotic-fed mice (PRO), antibiotics-treated mice (ATB) and mice that received combination of both probiotics and antibiotics (COMBIN). Microbiota from all treatment groups did not negatively affect viability of *C. difficile* after 1 h exposure since slight increase in growth was observed (Log_{10} CFU/mL variation: CTRL: +0.4, PRO: +0.1, ATB: +0.13, COMBIN: +0.09). Viability of *C. difficile* was then stable with minor fluctuations over time. While no mortality was noted (*i.e.* decrease in Log) when exposed to microbiota, data showed that growth occurring in late-exponential phase (*i.e.* by 3hrs time point) did not reached optimal levels in those groups, resulting in final total viable cells counts lower of

0.17, 0.25, 0.21 and 0.22 Log_{10} CFU/mL compared to the reference culture at the end of the experiment (5 hrs) for the CTRL, PRO, ATB and COMBIN groups respectively. However, from a biological significance point of view, we do not consider these differences in viability (<1 Log) as biologically relevant. Otherwise, we were not able to note a difference in *C. difficile* growth expansion or viability following exposure to a normal *versus* antibiotics-disrupted microbiota which is in contradiction with previous studies (Borriello & Barclay, 1986; Borriello et al., 1988; Nerandzic & Donskey, 2011).

It is important to point out that this model has some differences from the models seen in the studies presented above. First, in the present study, TY has been used as diluent for the microbiota suspensions. Since the experiment was directed towards the study of the QS system, any substantial inhibitory effect on *C. difficile* growth or viability would have been considered an indesirable effect. As QS is directly associated with population density, a high mortality rate could have influenced QS gene expression, with no regards of the states of the microbiota tested. We then used TY media instead of a nutrient-depleted media such as PBS or distilled water to minimize the potential inhibitory effect of the microbiota on *C. difficile* growth. Hence, it is likely that the nutrients contained in the TY media have promoted the growth and supported the viability of *C. difficile,* which could have been in opposition with the inhibitory effect of the microbiota. Otherwise, there is the possibility that the media has also selected for certain gut microbiota bacterial members that were not involved in the inhibitory effect on *C. difficile* and/or counter-selected for members involved in the inhibition. Finally, as a slight inhibitory effect on *C. difficile* growth expansion was measured nonetheless, it could be assumed that nutrients in the media were also consumed by members of the microbiota and that certain members were likely more fit to take and monopolize nutrients than *C. difficile,* which reduced nutrient availability in co-cultures.

Other differences that can explain why no significant growth inhibition was seen included the use of a high density *C. difficile* inoculum from a fit bacterial population (mid-exponential cells) and a relatively short experimental period of 5 hrs. Hence, the possibility that viability could have been more importantly impacted by using an extended experimental period, a smaller inoculum and/or by using test cells collected at earlier growth times, can not be excluded. Unfortunately, thoses scenarios were no tested in this study.

On the other hand, it has been demonstrated that acidic pH can inhibit *C. difficile* growth *in vitro.* When co-cultivated with lactobacillis, *C. difficile* growth is inhibited in media containing glucose such as brain heart infusion (BHI) but is unaffected in glucose-depleted media such as TY (Gunaratnam, Diarra, et al., 2021). Because of the use of TY in our model, it was unlikely that

important acidification occur in our co-cultures. However, we did not exclude beforehand this possibility and we monitored the pH during the experiment. As expected, the data suggested that acidification of the milieu could not have explained the growth limitation observed in this model. In fact, pH variations were negligible in co-cultures; CTRL: -0.21, PRO: -0.06, ATB: -0.10 and COMBIN: -0.24, and the larger pH variation occurred in the reference culture (-0.25). Furthermore, initial pH values measured immediately after the addition of microbiota (mean pH = 6.63 ± 0.14) and final pH values measured after 5 hrs of incubation (mean pH = 6.47 ± 0.04) stayed in the range of pH values measured *in vivo* in humans in the cecum and the colon (5.70-7.04) (Evans et al., 1988; Fallingborg, 1999).

5.3 *C. difficile* toxin production and gene expression in the presence of intestinal microbiota

In their colonisation resistance hamster model, Borriello *et al.* (1988) observed an increase in cytotoxic titre of 9 to 12 Log after 48 hours when *C. difficile* was grown in caecal emulsions of hamsters treated with clindamycin or ampicillin, while no toxin production was measured when hamsters were not treated with antibiotics (Borriello et al., 1988). Otherwise, it is well known that toxin production in *C. difficile* occurs in late stationary phase (Darkoh et al., 2015; Dupuy & Sonenshein, 1998; Hundsberger et al., 1997). Thus, it was suspected that the experiment time frame including at T1, the late exponential phase (8 hrs of growth) and at T5, what would correspond to the early stationary phase (12 hrs of growth), could not provide optimal and representative toxin production levels. Nonetheless, we were interested in investigating whether the presence of microbiota could stimulate a premature toxin production and if a disrupted microbiota would permit a more important toxin production than a normal microbiota. This question was inspired by the work of Darkoh *et al.* (2015) in which they were able to stimulate an early toxin production in *C. difficile*. By exposing low-density-exponential phase cells to a stationary phase culture supernatant, they were able to mimic a state of high-density population which promoted the cells to exhibit the quorum-dependant behavior prematurely. They determined that a solube signal (known today as the auto-induceur peptide (AIP) AgrD), secreted in the supernatant of the stationary phase culture, was responsible for the induction of toxin production. While it was unlikely that the microbiota could contain the AIP and further induce an early toxin production in our test-cells, we did not exclude the possibility that interaction between *C. difficile* and the microbiota and/or the presence of metabolites could stimulate an early production of toxins as well. It was then hypothesized that the onset of toxin synthesis could be prematured or delayed in the presence of normal or disrupted microbiota. In this present model, normal (CTRL) and probiotics supplemented (PRO) microbiota

failed to inhibit toxin production by *C. difficile,* which contradict previous studies. Additionnally, toxin A levels were slightly lower in dysbiotic microbiota (ATB) and dysbiotic microbiota supplemented in probiotics (COMBIN) compared to normal microbiota. While the difference in levels of toxin A was not significant, these observations are supported by the comparative gene expression analysis of *tcdA* and *tcdB.* When treatment groups (PRO, ATB and COMBIN) were compared to the CTRL microbiota, *tcdA* and *tcdB* expression appeared down-regulated in ATB and COMBIN groups (ATB: -5.56-fold, COMBIN: -12.5-fold) compared to CTRL group after 1 hours exposure, which is consistent with the lower levels of toxin A measured in the supernatant from the respective co-cultures.

It is important to note that toxin production and toxin genes expression were not monitored beyond the experiment time frame of this experiment (i.e., over 5 hrs exposure), which is a major divergence from other similar studies that measured toxin production beyond 24 hrs. It is not possible to exclude that toxin production trends would have demonstrated significant differences over a longer period as optimal toxin production in *C. difficile* is known to occur several hours after reaching stationary phase. Furthermore, this study design implies the sudden microbiota exposition to *C. difficile* bacteria from 7-hrs culture. Exposing *C. difficile* bacteria at earlier growth phase could have had a greater impact on toxin gene expression and production. Unfortunately, the effect of addition of microbiota in function of the bacteria growth phase was not investigated in this study. Otherwise, toxin B could not be monitored in the supernatants as levels did not reached the kit threshold. This was not surprising considering the evidence that toxin B is produced and secreted later than toxin A, which could explain why it was not detected during the experiment time frame of the current study (Dupuy & Sonenshein, 1998; Hundsberger et al., 1997).

In light of those results, it seems that the exposure of healthy and rich bacterial communities, found in normal and probiotics supplemented microbiota, did not resulted in lower toxin production than antibiotics-treated microbiota, where bacterial communities are expected to be negatively affected and be more permissive for toxin production. This study did not allow the exploration, at the cellular level, of the direct or indirect mechanisms possibly involved against or in the stimulation of *C. difficile* toxin synthesis but, it is clear that acidification was not involved in those mechanisms. Additionnaly, it is unlikely that gut bacteria could, by direct mechanisms, stimulated toxin synthesis in *C. difficile.* What it is more likely, however, is the implication of gut bacteria in the shaping of the gut environment. Virulence and in particular toxin synthesis, in *C. difficile* are known to be regulated by various environment-sensing regulators, such as global nutritional regulators CodY and CcpA. CodY and CcpA are implicated in the nutrient response of *C. difficile*

and participated in the regulation of *tcdA* and *tcdB* through the regulation of the toxin-specific sigma factor TcdR in response to nutrient and rapidly metabolizable carbon sources availability (A. Antunes et al., 2012; Antunes et al., 2011; Dineen et al., 2010; Dupuy & Sonenshein, 1998; Nawrocki et al., 2016). As availability of nutrient decreases, levels of branched-chain amino acids (BCAAs), GTP or glucose become limited which alleviated TcdR repression by CodY and CcpA, which in turns, stimulated *tcdA* and *tcdB* transcription and toxin synthesis. One way to explain the observations could be that microbiota from ATB and COMBIN mice were depleted in key members that normally occupy certain nutrient niches into the gastrointestinal tract, which results in an increased availability of nutrients. As explained above, virulence is tightly associated with the nutritional state of the bacterium. This could explain why in environment where nutrients are rapidly and efficiently utilized by the autochtonous bacteria, and in our case, in CTRL and PRO treatment groups, toxin synthesis is further de-repressed and higher levels of toxin production and *tcdA* and *tcdB* transcription can be observed in comparaison with nutrients-rich environments (see Figure 23).

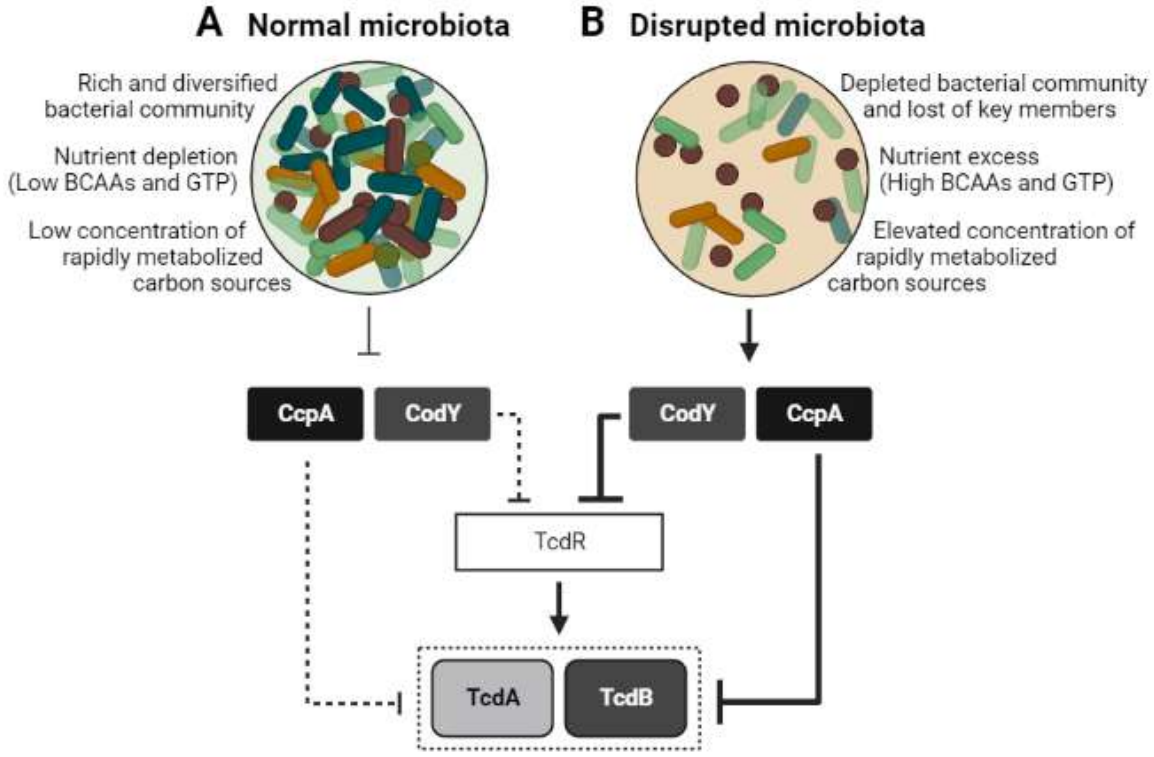

Figure 23. Proposed regulatory effect of microbiota condition on repression of TcdR and toxin transcription by transcriptional regulators CodY and CcpA in response of nutrient availability.

(A) In environment where nutrients availability is low such as in presence of rich and diversified microbiota, levels of branched-chain amino acids (BCAAs), GTP or glucose are limited. In this environment, global nutritional factors CodY and CcpA would be inactive, which would alleviate TcdR repression and stimulate toxin synthesis. (B) On the contrary, in environment where microbiota is disrupted and key members are lost, a higher availability

of nutrients would activate the global nutritional factors. Activation of CodY and CcpA would lead to the repression of TcdR or the direct repression of *tcdA* and *tcdB* transcription which would result in toxin synthesis inhibition.

Surprinsigly, toxin production in *C. difficile* exposed to normal microbiota compared to the toxin production measured in reference culture did not reflected *tcdA* and *tcdB* expression in time. While a rapid up-regulation of *tcdA* and *tcdB* expression after 1 hr occurred in presence of normal microbiota (CTRL), it was not associated with higher toxin A levels. By 3 and 5 hours, *tcdA* and *tcdB* were down-regulated in the CTRL group but then increased levels of toxin A were measured. It is plausible that the response to the up-regulation of toxin gene was delayed, as the up-regulation of *tcdA* at 1 hr could be responsible for the elevated toxin A synthesis and release measured by 3 hrs. However, it is still unclear why the up-regulation was not maintained beyond 1 hr.

Finally, the implication of the viable microbiota in the long term (beyond 3 hrs) on down-regulation of *tcdA* and *tcdB* as seen in the CTRL group compared to the reference culture is subject to discussion. Recent results demonstrated that a 4-fold decrease in *tcdA* expression was observed in *C. difficile* grown in "fecal water" made with BHI and that is deficient in viable microbiota compared to cells grown in BHI alone, suggesting on one hand, that presence of viable microbiota is not responsible solely for down-regulation of *tcdA* and on the other hand, supporting the potential role of environmental cues on toxin regulation. Furthermore, the investigator had proposed a potential association between the reduction in butyrate metabolism and the reduction in toxin stimulation (Moore, 2021).

5.4 Comparative analysis of QS genes in presence of microbiota

Little is known on how the QS systems in *C. difficile* are influenced during infection and what could be the role of microbiota in regulating those systems as a potential mechanism of colonization resistance. The *agr* QS system has been extensively studied in *S. aureus,* a Gram positive infectious agent involved in osteomyelitis (Lew & Waldvogel, 2004). The implication of *agr* in the regulation of virulence factors such as the δ-toxin and α-toxin in *S. aureus* is well established (Butrico & Cassat, 2020; Jenul & Horswill, 2019). In *C. difficile,* Martin *et al.* (2013) reported the implication of the *agr* locus in regulation of flagellar biosynthesis gene, c-di-GMP regulatory protein gene and *TcdA* gene, using a R20291 *agrA* mutant. In this study, the mutant showed significantly lower levels of TcdA production *in vitro* and down-regulation of *tcdA* transcript (Melissa J. Martin et al., 2013). Later, Darkoh *et al.* (2016) demonstrated that *C. difficile agr*1 locus, that harbors functional *agrB* and *agrD* genes, was essential for virulence as *agrB1D1* mutant could not produce toxin nor was

virulent in mice. The use of isogenic mutants in these studies have shed light onto the first evidence of virulence regulation by the *agr* QS system in *C. difficile.* Nonetheless, to our knowledge, none have investigated the impact of a more realistic and representative gut environment as wells as the microbiota on *C. difficile agr* QS system.

Taken into consideration that the *agr* system in *C. difficile* is likely involved at least in part in the bacterium virulence, we wanted to investigate the impact of the presence of microbiota on this QS system and to explore the singular effect of a normal *versus* a disrupted gut environment. To do so, we analysed the expression of the four *agr* genes; *agrA* and *agrC* located on the *agr*2 locus and *agrB* and *agrD* located on the *agr*1 locus, in reference culture and co-cultures of *C. difficile* and cecal and colonic content with various condition of microbiota. The results showed that all four *agr* genes were up-regulated after longer exposure to all microbiota (*i.e.* 3 and 5 hours) compared to the reference culture, while 1 hr of exposure did not resulted in appreciable change in gene expression. The up-regulation of the Agr system at the transcriptional level could not be explained by a stronger *C. difficile quorum* as growth was not stimulated in the presence of microbiota, suggesting that signals other than cell density, perhaps in the environment, have had an influence on *C. difficile* QS system. In *S. aureus,* various Agr regulators such as CodY, allow the bacterium to respond to extracellular environments signals in addition to AIP (Kavanaugh & Horswill, 2016; Majerczyk et al., 2010; Majerczyk et al., 2008; Roux et al., 2014). The work of Roux *et al.* (2014) demonstrated that AgrA can be activated following phosphorylation by AgrC even at low-cell density but that autoinduction of *agr* locus is prevented at these earlier stages by CodY indirect repression. Indeed, they discovered that the *agr* locus is expressed at early stage of exponential phase in *codY* null mutant (Roux et al., 2014). Those results suggested that activation of the *agr* locus is thightly controlled in response to environmental cues and is inhibited in a nutrient rich environment. From what we know, while there is evidence of toxin synthesis regulation by those transcriptional regulators, the possibility of *agr* locus regulation by those transcription regulators have not been explored in *C. difficile* yet.

When comparing the treatment groups (PRO, ATB and COMBIN) to CTRL group, the most important change in gene expression has occurred in the ATB group, where the four genes were over-expressed at 3 hrs (*agrA:* +1.97-fold, *agrC:* +1.57-fold, *agrB:* +5.11-fold, *agrD:* +4.74-fold) compared to CTRL microbiota. While gene expression of *agrA* and *agrC* showed great variability in time, important increase in fold-change of *agrB* and *agrD* occurred in the ATB group after 3 and 5 hours. Both transcripts were strongly up-regulated at 3 hrs (*agrB:* +5.11-fold, *agrD:* +4.74-fold) and 5 hrs (*agrB:* +6.37-fold, *agrD:* +7.84-fold) in the ATB group compared to CTRL group. Those

results contradict the previous findings in *S. aureus,* where *agr* locus activity is expected to be inhibited in nutrient-rich environment, assuming that the environment influenced by microbiota in the CTRL group is more depleted in rapidly metabolized nutrients than in the ATB group. However, numerous pieces of the puzzle are missing to fully understand the up-regulation of *agrBD* that was seen in the ATB and COMBIN groups but not in CTRL and PRO groups. First of all, it can only be assumed that nutrient availability is different in both normal and disrupted microbiota co-cultures as no metabolite analysis have been done on the cultures. Secondly, while growth conditions are manipulated to reproduce the anaerobic environment of the gut, *in vitro* culture of gut bacteria is difficult and more than often unsuccessful. Thus, it cannot be over seen that the isolation manipulations and the laboratory growth conditions had likely selected for specific members of the gut fitted to survived in those conditions, resulting firstly, in a biais of the representative microbiota exposed to *C. difficile,* and also, in a possible higher consumption of nutrients then anticipated.

As stated previously, the implication of QS and more precisely, AgrB and AgrD, in regulation of virulence in *C. difficile* have been established by Darkoh *et al.* (2015 & 2016). They showed that a deletion of *agr1* locus (containing functionnal *agrB* and *agrD* genes) in *C. difficile* 630 and R20291 resulted in the abolishement of toxins production, in the lost of cytotoxicity and avirulence in mice compared to the wild type strains. In the model used in the present study, when comparing the gene expression of the differents treatment groups together, it was demonstrated that *agrB* and *agrD* genes are strongly over-expressed when *C. difficile* is exposed to microbiota from antibiotics-treated mice (ATB and COMBIN) but not when exposed to normal microbiota (CTRL and PRO). A higher production of AgrB and AgrD could plays an important role in promoting virulence of *C. difficile* in those disrupted environments as we know that susceptibility to CDI in mammals is promoted by antibiotics consumption. However, this model was not able to link the elevated *agrBD* expression levels to a higher toxin production as TcdA levels were lower in ATB and COMBIN group despite the fact that *agrBD* transcription were strongly up-regulated. Nonetheless, the results could be explained, at least in part, by the observations of a recent study that demonstrated a distinct role for *agrB* and *agrD* in toxin production in *C. difficile* 630. The investigators constructed *agrB1, agrD1* and *agrB1D1* mutants and observed a 20-fold increase in *tcdA* and 12-fold increase in *tcdR* mRNA relative abundance in the *agrB1* mutant compared with the parent strain. The elevated transcription of *tcdA* was supported by increased protein levels (Ahmed et al., 2020). These results suggested a potential indirect repression of *tcdA* by AgrB which could involve the repression of *tcdR* transcription. The results of this present study seem to show a similar trend, where high transcription levels of *agrB* is associated with lower expression of *tcdA* and lower toxin production.

Furthermore, the reasons why a stronger and significant up-regulation was measured for *agrBD* and not for *agrAC* are still unknown. Because both gene groups; *agrD-agrB* and *agrC-agrA* are located on separated loci, it is likely that both groups are regulated independently. In *S. aureus,* it has been demonstrated that AgrA and AgrC are required for the *agr* hyperexpression phenotype in the CodY null mutant (Roux et al., 2014) which suggest their key role in the *agr* regulation. Because *agrACBD* genes are located on the same locus in *S. aureus* and are under control of the same promoter P2, it can be assumed that transcription levels of the *agrAC* and *agrBD* would be dependently regulated, resulting in similar expressions. Still, the presence of post-transcriptional regulation can not be excluded. Nonetheless, in the case of *C. difficile,* the results suggested that *agr1* and *agr2* loci are not co-regulated. Moreover, the absence of a putative two-components kinase system in the toxigenic *C. difficile* 630, which lacks the *agr2* locus that encode *agrA* and *agrC* (M. J. Martin et al., 2013), may suggest distinct roles of *agrBD* in *C. difficile* and explained the need of an independent regulation. Darkoh *et al.* (2013) described the essential role of the *agr1* locus in *C. difficile* pathogenesis. Yet, it remains unclear how the two-genes locus could regulate virulence in strain 630 without the two-component kinase system. Further work into establishing the potential specific regulators of *agrB1D1* and *agrA2C2* will definitely bring a better perspective of the regulatory network of *agr* QS system on virulence of *C. difficile.*

5.5 Impact of the probiotic supplementation

The model of this present study was set to investigate the impact of a normal and disrupted microbiota on growth, toxin production and QS and toxin gene expression. Another objective of this study was to investigate the effect of a specific probiotic formulation supplementation in both context of normal microbiota (PRO) and antibiotics-disrupted microbiota (COMBIN). Overall, the data showed that probiotics supplementation in mice (PRO) did not resulted in significant changes in terms of the microbiota's impact on *C. difficile* growth and viability, toxin production and gene expression compared with a normal microbiota (CTRL). Indeed, similar effects were seen between both groups for those parameters. This suggests either that the probiotics did not had a substantial influence on the microbiota which did not resulted in a significant impact on *C. difficile,* or that the probiotics did not have a specific inhibitory effect towards the pathogen regardless of its impact on the microbiota. Nonetheless, differences were noted in the case of *agrA* and *agrC* relative expressions, where both genes were under-expressed at all time in the PRO group compared to the CTRL group (*agrA:* mean -1.70-fold, SEM 0.42 and *agrC:* mean -2.25-fold, SEM 0.70). To our knowledge, potential inhibition of the QS pathways, either from inhibition of AIP binding by AgrC

or inhibition of transcriptional regulation by AgrA, in context of QS interference, have not been identified so far in *C. difficile*. However, the transcriptomic results suggested a possible negative regulation through the inhibition of the *agr2* locus (*agrA* and *agrC*) in presence of probiotics and/or probiotics-derived products.

Otherwise, important changes were seen at the transcriptional level in both *agrACBD* and toxin gene expressions between the microbiota from mice that received only antibiotics (ATB) and mice that received both antibiotics and probiotics (COMBIN). Differences in fold-change for *agrA* and *agrBD* between both groups were significant after 3 hrs. While demonstrating an increase in up-regulation of *agrB* and *agrD* after 5 hrs compared to 3 hrs in the COMBIN group, these fold-changes were still lower than the ones measured in the ATB group at 5 hrs. Interestingly, it seems that the presence of probiotics in the COMBIN group may have resulted in the alleviation of the over-expressions in the ATB group. Indeed, fold-change increase in *agrB* expression was lower after 3 hrs in the COMBIN group compared to ATB group (+1.12-*fold vs* +5.11-fold, $P \leq 0.05$) and again after 5 hrs (+4.30-fold *vs* +6.37-fold, $P = 0{,}43$). Similar results were obtained in fold change of *agrD* at 3 hrs exposure (-1.04-fold *vs* +4.74-fold, $P \leq 0.05$) and 5 hrs exposure (+4.80-fold *vs* +7.83-fold, $P = 0{,}34$). Moreover, significant fold-change of *agrA* was also observed at 3 hrs exposure in the COMBIN group where relative expression of the transcript was 2.33-fold lower compared to the relative expression in the ATB group ($P \leq 0.05$). Finally, while relative expression of *tcdA* and *tcdB* increased with time, both transcripts expression fold-change were lower in the COMBIN group compared to the ATB group at all time during the experiment. However, these results show not coincidence with the toxin production, as toxin A levels measured in both group were nearly identical and showed only a slight difference after 5 hrs, where toxin A level was higher in COMBIN group compared to ATB group.

As *agrBD* and toxin genes fold-change were generally decreased in COMBIN group compared to ATB group, a potential interfering effect by the probiotics is suggested. This effect may result from the global effect of the gut environment and microbiota shaped by the probiotic supplementation in the animals. The effect may also resulted from direct relation between probiotics and *C. difficile* in the co-cultures. Thus, further work is needed in order to determine if the probiotic interfering effect appeared before the isolation of the microbiota or if the viable probiotics had an active antagonistic effect against *C. difficile* in the co-cultures. Characterization of such acid-independent mechanism was beyond the scope of this study.

QS system interference by probiotics have been explored in various Gram-positive and Gram negative bacteria and have been reviewed recently (Gunaratnam, Millette, McFarland, DuPont, &

Lacroix, 2021). While effect of *agr* QS system on *C. difficile* virulence have been investigated, the impact of probiotics on the *agr* QS system is unknown. However, their impact on the LuxS/AI-2 QS system have been explored. Yun *et al.* (2014) investigated the effect of the strain *Lactobacillus acidophilus* GP1B on the QS system and toxin production in *C. difficile* RT 027. Using the AI-2 bioassay develop by Surette and Bassler (1998), they showed an inhibition in AI-2 production when *C. difficile* was grown in BHI media supplemented with increasing concentration of *L. acidophilus* GP1B cell extract (CE). In fact, at a concentration of 10 µg/mL, the CE induced a significant decrease in AI-2 production without affecting growth of *C. difficile.* Additionally, decrease in *txeR* (*tcdR*), *tcdA* and *tcdB* transcription have also be observed in presence of the probiotic CE (Yun, Oh, & Griffiths, 2014). In a similar study, Yong *et al* (2019) investigated the impact of *L. fermentum* Lim2 and showed similar trends with the previous study, with significant decrease in AI-2 production by *C. difficile* grown in presence of CE and decrease in transcriptional level of *luxS, tcdA* and *tcdB* as well as an increase in transcription of *tcdC,* a negative regulator of *tcdA* and *tcdB (Yong, Lim, Kim, Park, & Oh, 2019)*. While it has been proposed that the decrease in pathogenicity was associated with inhibition of the LuxS/AI-2 QS by the presence of probiotic CE in both study, this hypothesis could only be confirmed with the use of *C. difficile luxS* mutant. Because of this, and because investigation into the impact of probiotics on the *agr* QS system is lacking, further work is needed to truly appreciate to potential impact of probiotics in inhibition *C. difficile* virulence throught QS inhibition. To our knowledge, this present study was the first to explore the potential impact of a specific probiotic formulation on *C. difficile agr* QS system in a relevant environment, which seems to be acid-independent. However, subsequent investigation and model optimisation are needed to confirm and better understand the mechanisms involved in this effect.

6 CONCLUSION AND PERSPECTIVES

Current knowledge on the impact of the gut environment and microbiota on QS system of pathogens such as *C. difficile* and the subsequent impact on its virulence is limited. The use of various *agr* mutants have helped to better understand these regulatory networks (Ahmed et al., 2020; Darkoh et al., 2016; M. J. Martin et al., 2013) however, standard cultoromic approaches usually lack the adequate representation of the pathogen natural environmenent, the gut. Additionnally, interest in the potential role of microbiota in pathogen QS system interference as a potential mechanism of colonization resistance just begun to rise. In the case where QS signalling plays a crucial role in *C. difficile* virulence factors expression, interference with this system could represents an attractive approach as potential alternative to antibiotics in treatment and prevention of CDI. This work aimed to investigate the effect of normal, antibiotic-disrupted and probiotic-supplemented microbiota on *C. difficile agr* QS system and virulence. To do so, this project used an *ex vivo* model that enabled to directly target *C. difficile* QS activity modulation by the microbiota and to compare its impact according to its state.

First, a qRT-PCR assay was developed for the analysis of *agr* QS system in *C. difficile* in the purpose of providing reliable results and guaranting efficient and specific amplification of the target specimen genes in a highly complex bacterial environment. This assay enabled the separate analysis of the four *agr* genes and provided insights on their distinct regulation. Secondly, this investigation suggested that *agr* QS system activity is positively regulated in the presence of microbiota, underlying that *C. difficile* is indeed adaptating to its environment. This work also found that *agrB* and *agrD* genes are strongly up-regulated in presence of a disrupted gut environment and demonstrated an important disparity between up-regulation of *agrAC* and *agrBD* expressions, suggesting that independent regulations of both loci occur *in C. difficile*. Moreover, the results revealed a potential interference effect in both QS system and toxins transcription up-regulation that could be associated with the presence of probiotics in microbiota isolated from mice that received both antibiotic and probiotic treatments. By comparing *agr* and toxins gene expression and based on what is known in other Gram-positive pathogen such as *S. aureus,* the results of this present study could support the importance of the role of microbiota in the shaping of the gut environement, which could tightly link the nutrient environment, the QS and toxin production in *C. difficile.*

While this work have shed light onto the potential regulation of *C. difficile agr* QS system by the microbiota and the gut environment, severals questions remain unanswered. At the moment, it

is unknown how exactly the treatments (antibiotic and probiotic) have influenced the gut bacterial community in mice. DNA extraction have been performed on cecum and colonic content samples directly isolated from the animals as wells as on samples of the co-cultures at each time points in the *ex vivo* experiment, in order to perform a population analysis (16S RNAr). However, due to time constraints, those results could not be presented in this work as data acquisition and analysis have not been completed yet. Those results will confirm the microbiota disruption by the antibiotic treatment and will revealed the impact of the different treatments on the microbiota. Those analysis will also provide information about the capacity of the probiotic supplementation to beneficially alter the gut communities, which could help associating specific changes in the population with the alleviation of the indesirable impact of antibiotics. Otherwise, population analysis in the co-cultures will provide a better picture of the bacterial populations likely involved in the effects observed in this study.

As this work stood as a suitability experiment of the *ex vivo* model, it has provided useful insights on the biological variability of the samples and has raised some limitations and obstacles that are to be addressed in future experiments. Among them, is the fact that only a few replicates were included in this study (i.e., 3 replicates per group). Since this work demonstrated that an important biological variation exists within each individual group, a power analysis could be performed in order to estimate the appropriate sample size required to detect the effects of the various treatments. Otherwise, as the viability monitoring of the microbiota as well as the probiotics was not included in the experiment, it is unknown how those populations were affected. A potential way to monitor and confirm the viability of the microbiota and the probiotics could have been to plate the co-cultures on a variety of solid media such as the Bifidus selective medium (BSM), de Man de Rogosa and Sharpe medium (MRS), MacConkey medium (MAC), Violet red bile glucose medium (VRBG) and Columbia blood medium (CB). Those media target groups of bacteria like Bifidobacteria, acid lactic bacteria (LAB), Gram-negative bacteria and other enteric bacteria, Enterobacteriaceae and total bacteria respectively as it was described elsewhere (de Carvalho, Oliveira, Dib Saleh, Pintado, & Madureira, 2021). Moreover, the extracted RNA showed variable degrees of degradation (Figures 14-15). It was impossible to replicate the extraction because of limited sample volume. Considering the potential bias in gene expression results resulting from the use of bad quality RNA, and since several samples showed non optimal integrity, particular attention was put in the analysis of the amplification curves to ensure reproductibility between biological replicates for each group. Because amplifications of each gene showed similar Cq values between biological replicates regardless of their degradation status, all samples at all time points, excluding only one, have been included in the analysis. However, it is undeniable that the use of

intact and high-quality RNA remains the preferred practice in order to obtain robust and reliable gene expression data. Since the quality of RNA samples used in this experiment is not in accordance with MIQE guidelines, further work is needed in the optimization of the RNA extraction technique for highly complex samples such as those used in this study. In particular, the use of commercial RNA storage solutions such as RNAlater (Invitrogen) or RNAprotect (Qiagen) and the use of RNase inhibitors could be investigated to see whether those strategies could beneficially impact the integrity on the extracted RNA.

Finally, further investigation of the regulatory mechanisms potentially involved in the effect of microbiota on *C. difficile* QS and toxin production as seen in those experiments is needed. Further experiments could focus on the comparative analysis of transcriptional regulators involved in the nutrient response, such as *CodY* and *CcpA*, as well as other regulators of toxins gene expression: *TcdR* and *TcdC*. As suggested in the discussion, normal and antibiotic-disrupted microbiota must have shaped distinct nutrient environments, which could have impacted QS and toxin gene activity accordingly. Thus, having a more complete portrait of the environment, by measuring the concentration of carbons sources, BCAAs, SCFA and other metabolites could support the last hypothesis. In addition, the measurement of AIP production in the supernant could support the previous findings in which *agrD* (and *agrB*) were strongly up-regulated. Otherwise, as the LuxS QS system have not been studied in this project, it could be interesting to investiguated if this second QS system is modulated as well in presence of microbiota. AI-2 signalling represents what seems to be a universal inter-species communication and is proposed to be an important method for bacteria to senses their environment. The implication of this system in *C. difficile* virulence is controversial. However, even in the context where AI-2 produced by *C. difficile* is indeed just a by-product of the activated methyl cycle (Glen P Carter et al., 2005), the possibility that the bacterium could senses the environment it shares with other bacterial communities by sensing exogenous AI-2 and subsequently adapts its behavior can not be excluded (Pereira et al., 2013). Because of this, future gene expression analysis could also focus on the analysis of *luxS* to help decipher the implication of LuxS/AI-2 system in *C. difficile* virulence.

BIBLIOGRAPHY

Adams, D. A., Riggs, M. M., & Donskey, C. J. (2007). Effect of fluoroquinolone treatment on growth of and toxin production by epidemic and nonepidemic *Clostridium difficile* strains in the cecal contents of mice. *Antimicrob Agents Ch, 51*(8), 2674-2678. doi:10.1128/AAC.01582-06

Agrawal, N., Bettegowda, C., Cheong, I., Geschwind, J. F., Drake, C. G., Hipkiss, E. L., . . . Vogelstein, B. (2004). Bacteriolytic therapy can generate a potent immune response against experimental tumors. *Proc Natl Acad Sci U S A, 101*(42), 15172-15177. doi:10.1073/pnas.0406242101

Ahmed, U. K. B., Shadid, T. M., Larabee, J. L., & Ballard, J. D. (2020). Combined and distinct roles of Agr proteins in *Clostridioides difficile* 630 sporulation, motility, and toxin production. *mBio, 11*(6). doi:10.1128/mBio.03190-20

Al-Jumaili, I. J., Shibley, M., Lishman, A. H., & Record, C. O. (1984). Incidence and origin of *Clostridium difficile* in neonates. *J Clin Microbiol, 19*(1), 77-78. doi:10.1128/JCM.19.1.77-78.1984

Amabebe, E., Robert, F. O., Agbalalah, T., & Orubu, E. S. F. (2020). Microbial dysbiosis-induced obesity: role of gut microbiota in homoeostasis of energy metabolism. *Br J Nutr, 123*(10), 1127-1137. doi:10.1017/S0007114520000380

Antunes, A., Camiade, E., Monot, M., Courtois, E., Barbut, F., Sernova, N. V., . . . Dupuy, B. (2012). Global transcriptional control by glucose and carbon regulator CcpA in *Clostridium difficile*. *Nucleic Acids Res, 40*(21), 10701-10718. doi:10.1093/nar/gks864

Antunes, A., Martin-Verstraete, I., & Dupuy, B. (2011). CcpA-mediated repression of *Clostridium difficile* toxin gene expression. *Mol Microbiol, 79*(4), 882-899. doi:10.1111/j.1365-2958.2010.07495.x

Arumugam, M., Raes, J., Pelletier, E., Le Paslier, D., Yamada, T., Mende, D. R., . . . Bork, P. (2011). Enterotypes of the human gut microbiome. *Nature, 473*(7346), 174-180. doi:10.1038/nature09944

Awad, M. M., Johanesen, P. A., Carter, G. P., Rose, E., & Lyras, D. (2014). *Clostridium difficile* virulence factors: Insights into an anaerobic spore-forming pathogen. *Gut Microbes, 5*(5), 579-593. doi:10.4161/19490976.2014.969632

Backhed, F., Ley, R. E., Sonnenburg, J. L., Peterson, D. A., & Gordon, J. I. (2005). Host-bacterial mutualism in the human intestine. *Science, 307*(5717), 1915-1920. doi:10.1126/science.1104816

Barberio, B. A.-O., Facchin, S., Mele, E., D'Incà, R., Sturniolo, G. C., Farinati, F., . . . Savarino, E. A.-O. (2020). Faecal microbiota transplantation in *Clostridioides difficile* infection: real-life experience from an academic Italian hospital. *Therap Adv Gastroenterol.* doi: 10.1177/1756284820934315.

Bartlett, J. G. (1979). Antibiotic-associated pseudomembranous colitis. *Rev Infect Dis, 1*(3), 530-539. doi:10.1093/clinids/1.3.530

Bassler, B. L., Greenberg, E. P., & Stevens, A. M. (1997). Cross-species induction of luminescence in the quorum-sensing bacterium *Vibrio harveyi*. *J Bacteriol, 179*(12), 4043-4045. doi:10.1128/jb.179.12.4043-4045.1997

Bauer, M. P., Notermans, D. W., van Benthem, B. H., Brazier, J. S., Wilcox, M. H., Rupnik, M., . . . Kuijper, E. J. (2011). *Clostridium difficile* infection in Europe: a hospital-based survey. *Lancet, 377*(9759), 63-73. doi:10.1016/s0140-6736(10)61266-4

Best, E. L., Freeman, J., & Wilcox, M. H. (2012). Models for the study of *Clostridium difficile* infection. *Gut microbes, 3*(2), 145-167. doi:10.4161/gmic.19526

Bilverstone, T. W., Garland, M., Cave, R. J., Kelly, M. L., Tholen, M., Bouley, D. M., . . . Melnyk, R. A. (2020). The glucosyltransferase activity of *C. difficile* Toxin B is required for disease pathogenesis. *PLoS Pathog, 16*(9), e1008852. doi:10.1371/journal.ppat.1008852

Birnbaum, J., Bartlett, J. G., & Gelber, A. C. (2008). *Clostridium difficile*: an under-recognized cause of reactive arthritis? *Clin Rheumatol, 27*(2), 253-255. doi:10.1007/s10067-007-0710-2

Borriello, S. P., & Barclay, F. E. (1986). An in-vitro model of colonisation resistance to *Clostridium difficile* infection. *J Med Microbiol, 21*(4), 299-309. doi:10.1099/00222615-21-4-299

Borriello, S. P., Barclay, F. E., & Welch, A. R. (1988). Evaluation of the predictive capability of an in-vitro model of colonisation resistance to *Clostridium difficile* infection. *Microb Ecol Health D, 1*(1), 61-64. doi:10.3109/08910608809140181

Brandt, L. J., Aroniadis, O. C., Mellow, M., Kanatzar, A., Kelly, C., Park, T., . . . Surawicz, C. (2012). Long-Term Follow-Up of Colonoscopic Fecal Microbiota Transplant for Recurrent *Clostridium difficile* Infection. *Am J Gastroenterol, 107*(7), 1079-1087. doi:10.1038/ajg.2012.60

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., . . . Wittwer, C. T. (2009). The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem, 55*(4), 611-622. doi:10.1373/clinchem.2008.112797

Butrico, C. E., & Cassat, J. E. (2020). *Quorum Sensing* and toxin production in *Staphylococcus aureus* osteomyelitis: Pathogenesis and paradox. *Toxins (Basel), 12*(8). doi:10.3390/toxins12080516

C Pothoulakis, L. M. B., R Ely, B Faris, M E Clark, C Franzblau, J T LaMont. (1986). Purification and properties of *Clostridium difficile* cytotoxin B. *J Biol Chem, 261*(January 25), 1316-1321.

Caballero-Franco, C., Keller, K., De Simone, C., Chadee, K. (2007). The VSL#3 probiotic formula induces mucin gene expression and secretion in colonic epithelial cells. *Am J Physiol Gastrointest Liver Physiol*, *292*(1), G315-22. doi: 10.1152/ajpgi.00265.2006.

Carter, A. T., & Peck, M. W. (2015). Genomes, neurotoxins and biology of *Clostridium botulinum* Group I and Group II. *Res Microbiol, 166*(4), 303-317. doi:10.1016/j.resmic.2014.10.010

Carter, G. P., Chakravorty, A., Pham Nguyen, T. A., Mileto, S., Schreiber, F., Li, L., . . . Lyras, D. (2015). Defining the roles of TcdA and TcdB in localized gastrointestinal disease, systemic organ damage, and the host response during *Clostridium difficile* infections. *mBio, 6*(3), e00551. doi:10.1128/mBio.00551-15

Carter, G. P., Purdy, D., Williams, P., & Minton, N. P. (2005). *Quorum sensing* in *Clostridium difficile*: analysis of a luxS-type signalling system. *J Med Microbiol, 54*(2), 119-127. doi:https://doi.org/10.1099/jmm.0.45817-0

Chandrasekaran, R., & Lacy, D. B. (2017). The role of toxins in *Clostridium difficile* infection. *FEMS Microbiol Rev, 41*(6), 723-750. doi:10.1093/femsre/fux048

Chapeton-Montes, D., Plourde, L., Deneve, C., Garnier, D., Barbirato, F., Colombié, V., . . . Popoff, M. R. (2020). Tetanus toxin synthesis is under the control of a complex network of regulatory genes in *Clostridium tetani*. *Toxins (Basel), 12*(5). doi:10.3390/toxins12050328

Chen, Katchar, K., Goldsmith, J. D., Nanthakumar, N., Cheknis, A., Gerding, D. N., & Kelly, C. P. (2008). A mouse model of *Clostridium difficile*-associated disease. *Gastroenterology, 135*(6), 1984-1992. doi:10.1053/j.gastro.2008.09.002

Chen, J., Rood, J. I., & McClane, B. A. (2011). Epsilon-Toxin production by genus-species *Clostridium perfringens* Type D strain CN3718 is dependent upon the operon but not the VirS/VirR two-component regulatory system. *mBio, 2*(6), e00275-00211. doi:10.1128/mBio.00275-11

Collignon, A., Ticchi, L., Depitre, C., Gaudelus, J., Delmée, M., & Corthier, G. (1993). Heterogeneity of *Clostridium difficile* isolates from infants. *Eur J Pediatr, 152*(4), 319-322. doi:10.1007/bf01956743

Cooksley, C. M., Davis, I. J., Winzer, K., Chan, W. C., Peck, M. W., & Minton, N. P. (2010). Regulation of neurotoxin production and sporulation by a Putative agrBD signaling system in proteolytic *Clostridium botulinum*. *Appl Environ Microbiol, 76*(13), 4448-4460. doi:10.1128/aem.03038-09

Cowardin, C. A., Buonomo, E. L., Saleh, M. M., Wilson, M. G., Burgess, S. L., Kuehne, S. A., . . . Petri, W. A., Jr. (2016). The binary toxin CDT enhances *Clostridium difficile* virulence by suppressing protective colonic eosinophilia. *Nat Microbiol, 1*(8), 16108. doi:10.1038/nmicrobiol.2016.108

Cox, A. D., St Michael, F., Aubry, A., Strong, P. C. R., Hayes, A. C., & Logan, S. M. (2020). Comparison of polysaccharide glycoconjugates as candidate vaccines to combat *Clostridiodes (Clostridium) difficile*. *Glycoconj J*. doi:10.1007/s10719-020-09937-9

Crobach, M. J. T., Vernon, J. J., Loo, V. G., Kong, L. Y., Pechine, S., Wilcox, M. H., & Kuijper, E. J. (2018). Understanding *Clostridium difficile* colonization. *Clin Microbiol Rev, 31*(2). doi:10.1128/CMR.00021-17

Crowther, G. S., Chilton, C. H., Todhunter, S. L., Nicholson, S., Freeman, J., Baines, S. D., & Wilcox, M. H. (2014). Development and validation of a chemostat gut model to study both planktonic and biofilm modes of growth of *Clostridium difficile* and human microbiota. *PLoS One, 9*(2), e88396. doi:10.1371/journal.pone.0088396

Cruz-Morales, P., Orellana, C. A., Moutafis, G., Moonen, G., Rincon, G., Nielsen, L. K., & Marcellin, E. (2019). Revisiting the Evolution and Taxonomy of Clostridia, a Phylogenomic Update. *Genome Biol Evol, 11*(7), 2035-2044. doi:10.1093/gbe/evz096

Czepiel, J., Drozdz, M., Pituch, H., Kuijper, E. J., Perucki, W., Mielimonka, A., . . . Biesiada, G. (2019). *Clostridium difficile* infection: review. *Eur J Clin Microbiol Infect Dis, 38*(7), 1211-1221. doi:10.1007/s10096-019-03539-6

Dang, L. H., Bettegowda, C., Agrawal, N., Cheong, I., Huso, D., Frost, P., . . . Vogelstein, B. (2004). Targeting vascular and avascular compartments of tumors with *C. novyi*-NT and anti-microtubule agents. *Cancer Biol Ther, 3*(3), 326-337. doi:10.4161/cbt.3.3.704

Dang, L. H., Bettegowda, C., Huso, D. L., Kinzler, K. W., & Vogelstein, B. (2001). Combination bacteriolytic therapy for the treatment of experimental tumors. *Proc Natl Acad Sci U S A, 98*(26), 15155-15160. doi:10.1073/pnas.251543698

Dansinger, M. L., Johnson, S., Jansen, P. C., Opstad, N. L., Bettin, K. M., & Gerding, D. N. (1996). Protein-losing enteropathy is associated with *Clostridium difficile* diarrhea but not with asymptomatic colonization: a prospective, case-control study. *Clin Infect Dis, 22*(6), 932-937. doi:10.1093/clinids/22.6.932

Đapa, T., Leuzzi, R., Ng, Y. K., Baban, S. T., Adamo, R., Kuehne, S. A., . . . Unnikrishnan, M. (2013). Multiple factors modulate biofilm formation by the anaerobic pathogen *Clostridium difficile*. *J Bacteriol, 195*(3), 545-555. doi:10.1128/jb.01980-12

Darkoh, C., DuPont, H. L., Norris, S. J., & Kaplan, H. B. (2015). Toxin synthesis by *Clostridium difficile* is regulated through quorum signaling. *mBio, 6*(2), e02569. doi:10.1128/mBio.02569-14

Darkoh, C., Odo, C., & DuPont, H. L. (2016). Accessory Gene Regulator-1 locus is essential for virulence and pathogenesis of *Clostridium difficile*. *mBio, 7*(4), e01237-01216. doi:10.1128/mBio.01237-16

de Carvalho, N. M., Oliveira, D. L., Dib Saleh, M. A., Pintado, M., & Madureira, A. R. (2021). Preservation of human gut microbiota inoculums for *in vitro* fermentations studies. *Fermentation, 7*(1), 14. doi.org/10.3390/fermentation7010014

de Kievit, T. R., & Iglewski, B. H. (2000). Bacterial *quorum sensing* in pathogenic relationships. *Infect Immun, 68*(9), 4839-4849. doi:10.1128/iai.68.9.4839-4849.2000

Deep, A., Chaudhary, U., & Gupta, V. (2011). *Quorum sensing* and bacterial pathogenicity: From molecules to disease. *J Lab Physicians, 3*(1), 4-11. doi:10.4103/0974-2727.78553

Dineen, S. S., McBride, S. M., & Sonenshein, A. L. (2010). Integration of metabolism and virulence by *Clostridium difficile* CodY. *J Bacteriol, 192*(20), 5350-5362. doi:10.1128/JB.00341-10

Donskey, C. J., Kundrapu, S., & Deshpande, A. (2015). Colonization versus carriage of *Clostridium difficile*. *Infect Dis Clin North Am, 29*(1), 13-28. doi:10.1016/j.idc.2014.11.001

Dowle, C. (2016). Faecal microbiota transplantation: a review of FMT as an alternative treatment for *Clostridium difficile* infection. *Bioscience Horizons, 9*. doi:10.1093/biohorizons/hzw007

Drake, D. R. B., K. A. (2002). Chapter 2. Continuous-culture chemostat systems and flowcells as methods to investigate microbial interactions (K. A. G. Brogden, J. M. Ed.). Washington (DC): ASM.

Dubberke, E. R., Reske, K. A., Olsen, M. A., McDonald, L. C., & Fraser, V. J. (2008). Short- and long-term attributable costs of *Clostridium difficile*-associated disease in nonsurgical inpatients. *Clin Infect Dis, 46*(4), 497-504. doi:10.1086/526530

Dubois, N. E., Read, C. Y., O'Brien, K., & Ling, K. (2020). Challenges of screening prospective stool donors for fecal microbiota transplantation. *Biol Res Nurs*, *23*(1), 21-30. doi:10.1177/1099800420941185

Dupuy, B., & Sonenshein, A. L. (1998). Regulated transcription of *Clostridium difficile* toxin genes. *Mol Microbiol, 27*(1), 107-120. doi:10.1046/j.1365-2958.1998.00663.x

Ebrahimzadeh Leylabadlo, H., Sanaie, S., Sadeghpour Heravi, F., Ahmadian, Z., & Ghotaslou, R. (2020). From role of gut microbiota to microbial-based therapies in type 2-diabetes. *Infect Genet Evol, 81*, 104268. doi:10.1016/j.meegid.2020.104268

Elvers, K. T., & Park, S. F. (2002). *Quorum sensing* in *Campylobacter jejuni*: detection of a luxS encoded signalling molecule. *Microbiology (Reading), 148*(Pt 5), 1475-1481. doi:10.1099/00221287-148-5-1475

Endt, K., Stecher, B., Chaffron, S., Slack, E., Tchitchek, N., Benecke, A., . . . Hardt, W. D. (2010). The microbiota mediates pathogen clearance from the gut lumen after non-typhoidal *Salmonella* diarrhea. *PLoS Pathog, 6*(9), e1001097. doi:10.1371/journal.ppat.1001097

Evans, D. F., Pye, G., Bramley, R., Clark, A. G., Dyson, T. J., & Hardcastle, J. D. (1988). Measurement of gastrointestinal pH profiles in normal ambulant human subjects. *Gut, 29*(8), 1035-1041. doi:10.1136/gut.29.8.1035

Fachi, J. L., Felipe, J. S., Pral, L. P., da Silva, B. K., Corrêa, R. O., de Andrade, M. C. P., . . . Vinolo, M. A. R. (2019). Butyrate protects mice from *Clostridium difficile*-induced colitis through an HIF-1-dependent mechanism. *Cell Rep, 27*(3), 750-761 e757. doi:10.1016/j.celrep.2019.03.054

Fallingborg, J. (1999). Intraluminal pH of the human gastrointestinal tract. *Dan Med Bull, 46*(3), 183-196.

Finn, E., Andersson, F. L., & Madin-Warburton, M. (2021). Burden of *Clostridioides difficile* infection (CDI) - a systematic review of the epidemiology of primary and recurrent CDI. *BMC Infect Dis, 21*(1), 456. doi:10.1186/s12879-021-06147-y

Flynn, S., van Sinderen, D., Thornton, G. M., Holo, H., Nes, I. F., & Collins, J. K. (2002). Characterization of the genetic locus responsible for the production of ABP-118, a novel bacteriocin produced by the probiotic bacterium *Lactobacillus salivarius* subsp. *salivarius* UCC118. *Microbiology (Reading), 148*(Pt 4), 973-984. doi:10.1099/00221287-148-4-973

Freeman, J., O'Neill, F. J., & Wilcox, M. H. (2003). Effects of cefotaxime and desacetylcefotaxime upon *Clostridium difficile* proliferation and toxin production in a triple-stage chemostat model of the human gut. *J Antimicrob Chemoth, 52*(1), 96-102. doi:10.1093/jac/dkg267

Gagniere, J., Raisch, J., Veziant, J., Barnich, N., Bonnet, R., Buc, E., . . . Bonnet, M. (2016). Gut microbiota imbalance and colorectal cancer. *World J Gastroenterol, 22*(2), 501-518. doi:10.3748/wjg.v22.i2.501

Gaisawat, M. B., Iskandar, M. M., MacPherson, C. W., Tompkins, T. A., & Kubow, S. (2019). Probiotic supplementation is associated with increased antioxidant capacity and copper chelation in *C. difficile*-infected fecal water. *Nutrients, 11*(9). doi:10.3390/nu11092007

Gaisawat, M. B., MacPherson, C. W., Tremblay, J., Piano, A., Iskandar, M. M., Tompkins, T. A., & Kubow, S. (2019). Probiotic supplementation in a *Clostridium difficile*-infected gastrointestinal model is sssociated with restoring metabolic function of microbiota. *Microorganisms, 8*(1). doi:10.3390/microorganisms8010060

George, R. H., Symonds, J. M., Dimock, F., Brown, J. D., Arabi, Y., Shinagawa, N., . . . Burdon, D. W. (1978). Identification of *Clostridium difficile* as a cause of pseudomembranous colitis. *Br Med J, 1*(6114), 695. doi:10.1136/bmj.1.6114.695

Gerding, D. N. (2004). Clindamycin, cephalosporins, fluoroquinolones, and *Clostridium difficile*-associated diarrhea: this is an antimicrobial resistance problem. *Clin Infect Dis, 38*(5), 646-648. doi:10.1086/382084

Ghimire, S., Roy, C., Wongkuna, S., Antony, L., Maji, A., Keena, M. C., . . . Scaria, J. (2020). Identification of *Clostridioides difficile* inhibiting gut commensals using culturomics, phenotyping, and combinatorial community assembly. *mSystems, 5*(1), e00620-00619. doi:10.1128/mSystems.00620-19

Giles, E. M., D'Adamo, G. L., & Forster, S. C. (2019). The future of faecal transplants. *Nat Rev Microbiol, 17*(12), 719. doi:10.1038/s41579-019-0271-9

Girinathan, B. P., DiBenedetto, N., Worley, J., Peltier, J., Lavin, R., Delaney, M. L., . . . Bry, L. (2020). The mechanisms of *in vivo* commensal control of *Clostridioides difficile* virulence. *Cell Host Microbe,* doi:10.1101/2020.01.04.894915

Girón, J. A., Torres, A. G., Freer, E., & Kaper, J. B. (2002). The flagella of enteropathogenic *Escherichia coli* mediate adherence to epithelial cells. *Mol Microbiol, 44*(2), 361-379. doi:10.1046/j.1365-2958.2002.02899.x

Gray, B., Hall, P., & Gresham, H. (2013). Targeting agr- and agr-Like *quorum sensing* systems for development of common therapeutics to treat multiple gram-positive bacterial infections. *Sensors (Basel), 13*(4), 5130-5166. doi:10.3390/s130405130

Guh, A. Y., Mu, Y., Winston, L. G., Johnston, H., Olson, D., Farley, M. M., . . . McDonald, L. C. (2020). Trends in U.S. Burden of *Clostridioides difficile* infection and outcomes. *N Engl J Med, 382*(14), 1320-1330. doi:10.1056/NEJMoa1910215

Guinane, C. M., Lawton, E. M., O'Connor, P. M., O'Sullivan, O., Hill, C., Ross, R. P., & Cotter, P. D. (2016). The bacteriocin bactofencin A subtly modulates gut microbial populations. *Anaerobe, 40*, 41-49. doi:10.1016/j.anaerobe.2016.05.001

Gunaratnam, S., Diarra, C., Paquette, P. D., Ship, N., Millette, M., & Lacroix, M. (2021). The acid-dependent and independent effects of *Lactobacillus acidophilus* CL1285, *Lacticaseibacillus casei* LBC80R, and *Lacticaseibacillus rhamnosus* CLR2 on *Clostridioides difficile* R20291. *Probiotics Antimicrob Proteins, 13*(4), 949-956. doi:10.1007/s12602-020-09729-5

Gunaratnam, S., Millette, M., McFarland, L. V., DuPont, H. L., & Lacroix, M. (2021). Potential role of probiotics in reducing *Clostridioides difficile* virulence: Interference with *quorum sensing* systems. *Microb Pathog, 153*, 104798. doi:10.1016/j.micpath.2021.104798

Hammer, B. K., & Bassler, B. L. (2003). *Quorum sensing* controls biofilm formation in *Vibrio cholerae*. *Mol Microbiol, 50*(1), 101-104. doi:10.1046/j.1365-2958.2003.03688.x

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., . . . Sanders, M. E. (2014). Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat Rev Gastroenterol Hepatol, 11*(8), 506-514. doi:10.1038/nrgastro.2014.66

Hookman, P., & Barkin, J. S. (2009). *Clostridium difficile* associated infection, diarrhea and colitis. *World J Gastroenterol, 15*(13), 1554-1580. doi:10.3748/wjg.15.1554

Hooks, K. B., & O'Malley, M. A. (2017). Dysbiosis and its discontents. *mBio, 8*(5). doi:10.1128/mBio.01492-17

Hundsberger, T., Braun, V., Weidmann, M., Leukel, P., Sauerborn, M., & von Eichel-Streiber, C. (1997). Transcription analysis of the genes tcdA-E of the pathogenicity locus of *Clostridium difficile*. *Eur J Biochem, 244*(3), 735-742. doi:10.1111/j.1432-1033.1997.t01-1-00735.x

Janezic, S., Marín, M., Martín, A., & Rupnik, M. (2015). A new type of toxin A-negative, toxin B-positive *Clostridium difficile* strain lacking a complete *tcdA* gene. *J Clin Microbiol, 53*(2), 692-695. doi:10.1128/jcm.02211-14

Jangi, S., & Lamont, J. T. (2010). Asymptomatic colonization by *Clostridium difficile* in infants: implications for disease in later life. *J Pediatr Gastroentero Nutr, 51*(1), 2-7. doi: 10.1097/MPG.0b013e3181d29767.

Jenior, M. L., Leslie, J. L., Young, V. B., & Schloss, P. D. (2017). *Clostridium difficile* colonizes alternative nutrient niches during infection across distinct murine gut microbiomes. *mSystems, 2*(4). doi:10.1128/mSystems.00063-17

Jenul, C., & Horswill, A. R. (2019). Regulation of S*taphylococcus aureus* virulence. *Microbiol Spectr, 7*(2). doi:10.1128/microbiolspec.GPP3-0031-2018

Johnson, S., Lavergne, V., Skinner, A. M., Gonzales-Luna, A. J., Garey, K. W., Kelly, C. P., & Wilcox, M. H. (2021). Clinical Practice Guideline by the Infectious Diseases Society of America (IDSA) and Society for Healthcare Epidemiology of America (SHEA): 2021 Focused update guidelines on management of *Clostridioides difficile* infection in adults. *Clin Infect Dis, 73*(5), e1029-e1044. doi:10.1093/cid/ciab549

Juul, F. E., Garborg, K., Bretthauer, M., Skudal, H., Oines, M. N., Wiig, H., . . . Adami, H. O. (2018). Fecal microbiota transplantation for primary *Clostridium difficile* infection. *N Engl J Med, 378*(26), 2535-2536. doi:10.1056/NEJMc1803103

Katz, K. C., Golding, G. R., Choi, K. B., Pelude, L., Amaratunga, K. R., Taljaard, M., . . . Canadian Nosocomial Infection Surveillance, P. (2018). The evolving epidemiology of *Clostridium difficile* infection in Canadian hospitals during a postepidemic period (2009-2015). *CMAJ, 190*(25), E758-E765. doi:10.1503/cmaj.180013

Kavanaugh, J. S., & Horswill, A. R. (2016). Impact of Environmental Cues on Staphylococcal *Quorum Sensing* and biofilm development. *J Biol Chem, 291*(24), 12556-12564. doi:10.1074/jbc.R116.722710

Khattab, R. A., Ahmed, N. A., Ragab, Y. M., & Rasmy, S. A. (2020). Bacteria producing antimicrobials against *Clostridium difficile* isolated from human stool. *Anaerobe, 63*, 102206. doi:10.1016/j.anaerobe.2020.102206

Kim, J., Kim, Y., & Pai, H. (2016). Clinical characteristics and treatment outcomes of *Clostridium difficile* infections by PCR Ribotype 017 and 018 Strains. *PLoS One, 11*(12), e0168849. doi:10.1371/journal.pone.0168849

Krutova, M., Nyc, O., Matejkova, J., Kuijper, E. J., Jalava, J., & Mentula, S. (2018). The recognition and characterisation of Finnish *Clostridium difficile* isolates resembling PCR-ribotype 027. *J Microbiol Immunol Infect, 51*(3), 344-351. doi:10.1016/j.jmii.2017.02.002

Kuehne, S. A., Cartman, S. T., Heap, J. T., Kelly, M. L., Cockayne, A., & Minton, N. P. (2010). The role of toxin A and toxin B in *Clostridium difficile* infection. *Nature, 467*(7316), 711-713. doi:10.1038/nature09397

Kukla, M., Adrych, K., Dobrowolska, A., Mach, T., Regula, J., & Rydzewska, G. (2020). Guidelines for *Clostridium difficile* infection in adults. *Prz Gastroenterol, 15*(1), 1-21. doi:10.5114/pg.2020.93629

Lagier, J.-C. (2016). Gut microbiota and *Clostridium difficile* infections. *Human Microbiome Journal, 2*, 10-14. doi:https://doi.org/10.1016/j.humic.2016.10.003

Larson, H. E., Price, A. B., Honour, P., & Borriello, S. P. (1978). *Clostridium Difficile* and the aetiology of pseudomembranous colitis. *The Lancet, 311*(8073), 1063-1066. doi:10.1016/s0140-6736(78)90912-1

Lawley, T. D., & Walker, A. W. (2013). Intestinal colonization resistance. *Immunology, 138*(1), 1-11. doi:https://doi.org/10.1111/j.1365-2567.2012.03616.x

Leal, J. R., Heitman, S. J., Conly, J. M., Henderson, E. A., & Manns, B. J. (2016). Cost-effectiveness Analysis of the use of probiotics for the prevention of *Clostridium difficile*-associated diarrhea in a provincial healthcare system. *Infect Control Hosp Epidemiol, 37*(9), 1079-1086. doi:10.1017/ice.2016.134

Lee, A. S., & Song, K. P. (2005). LuxS/autoinducer-2 *quorum sensing* molecule regulates transcriptional virulence gene expression in *Clostridium difficile*. *Biochem Biophys Res Commun, 335*(3), 659-666. doi:10.1016/j.bbrc.2005.07.131

Lee, J., Jang, Y.-S., Choi, S. J., Im, J. A., Song, H., Cho, J. H., . . . Lee, S. Y. (2012). Metabolic engineering of *Clostridium acetobutylicum* ATCC 824 for isopropanol-butanol-ethanol fermentation. *Appl Environ Microb, 78*(5), 1416-1423. doi:10.1128/aem.06382-11

Leffler, D. A., & Lamont, J. T. (2015). *Clostridium difficile* Infection. *New Engl J Med, 372*(16), 1539-1548. doi:10.1056/NEJMra1403772

Lessa, F. C., Mu, Y., Bamberg, W. M., Beldavs, Z. G., Dumyati, G. K., Dunn, J. R., . . . McDonald, L. C. (2015). Burden of *Clostridium difficile* infection in the United States. *N Engl J Med, 372*(9), 825-834. doi:10.1056/NEJMoa1408913

Lew, D. P., & Waldvogel, F. A. (2004). Osteomyelitis. *The Lancet, 364*(9431), 369-379. doi:10.1016/s0140-6736(04)16727-5

Li, S., Huang, L., Ke, C., Pang, Z., & Liu, L. (2020). Pathway dissection, regulation, engineering and application: lessons learned from biobutanol production by solventogenic clostridia. *Biotechnol Biofuels, 13*, 39. doi:10.1186/s13068-020-01674-3

Litvak, Y., & Baumler, A. J. (2019). The founder hypothesis: A basis for microbiota resistance, diversity in taxa carriage, and colonization resistance against pathogens. *PLoS Pathog, 15*(2), e1007563. doi:10.1371/journal.ppat.1007563

Liu, S. C., Ahn, G. O., Kioi, M., Dorie, M. J., Patterson, A. V., & Brown, J. M. (2008). Optimized clostridium-directed enzyme prodrug therapy improves the antitumor activity of the novel DNA cross-linking agent PR-104. *Cancer Res, 68*(19), 7995-8003. doi:10.1158/0008-5472.Can-08-1698

Liu, S. C., Minton, N. P., Giaccia, A. J., & Brown, J. M. (2002). Anticancer efficacy of systemically delivered anaerobic bacteria as gene therapy vectors targeting tumor hypoxia/necrosis. *Gene Ther, 9*(4), 291-296. doi:10.1038/sj.gt.3301659

Louis, P., & Flint, H. J. (2017). Formation of propionate and butyrate by the human colonic microbiota. *Environ Microbiol, 19*(1), 29-41. doi:10.1111/1462-2920.13589

Lyras, D., O'Connor, J. R., Howarth, P. M., Sambol, S. P., Carter, G. P., Phumoonna, T., . . . Rood, J. I. (2009). Toxin B is essential for virulence of *Clostridium difficile*. *Nature, 458*(7242), 1176-1179. doi:10.1038/nature07822

MacCannell, D. R., Louie, T. J., Gregson, D. B., Laverdiere, M., Labbe, A. C., Laing, F., & Henwick, S. (2006). Molecular analysis of *Clostridium difficile* PCR ribotype 027 isolates from Eastern and Western Canada. *J Clin Microbiol, 44*(6), 2147-2152. doi:10.1128/JCM.02563-05

Macfarlane, G. T., Macfarlane, S., & Gibson, G. R. (1998). Validation of a Three-Stage compound continuous culture system for investigating the effect of retention time on the ecology and metabolism of bacteria in the human colon. *Microb Ecol, 35*(2), 180-187. doi:10.1007/s002489900072

Mack, D. R., Michail, S., Wei, S., McDougall, L., & Hollingsworth, M. A. (1999). Probiotics inhibit enteropathogenic *E. coli* adherence in vitro by inducing intestinal mucin gene expression. *Am J Physiol, 276*(4), G941-950. doi:10.1152/ajpgi.1999.276.4.G941

Magill, S. S., Edwards, J. R., Bamberg, W., Beldavs, Z. G., Dumyati, G., Kainer, M. A. (2014). Multistate point-prevalence survey of health care-associated infections. *N Engl J Med, 370*(13), 1198-1208. doi:10.1056/NEJMoa1306801

Majerczyk, C. D., Dunman, P. M., Luong, T. T., Lee, C. Y., Sadykov, M. R., Somerville, G. A., . . . Sonenshein, A. L. (2010). Direct targets of CodY in *Staphylococcus aureus*. *J Bacteriol, 192*(11), 2861-2877. doi:10.1128/JB.00220-10

Majerczyk, C. D., Sadykov, M. R., Luong, T. T., Lee, C., Somerville, G. A., & Sonenshein, A. L. (2008). *Staphylococcus aureus* CodY negatively regulates virulence gene expression. *J Bacteriol, 190*(7), 2257-2265. doi:10.1128/JB.01545-07

Martin, M. J., Clare, S., Goulding, D., Faulds-Pain, A., Barquist, L., Browne, H. P., . . . Wren, B. W. (2013). The agr locus regulates virulence and colonization genes in *Clostridium difficile* 027. *J Bacteriol, 195*(16), 3672-3681. doi:10.1128/jb.00473-13

Marvaud, J. C., Quevedo-Torres, S., Eckert, C., Janoir, C., & Barbut, F. (2019). Virulence of new variant strains of *Clostridium difficile* producing only toxin A or binary toxin in the hamster model. *New Microbes New Infect, 32*, 100590. doi:10.1016/j.nmni.2019.100590

Mathur, H., Rea, M. C., Cotter, P. D., Ross, R. P., & Hill, C. (2014). The potential for emerging therapeutic options for *Clostridium difficile* infection. *Gut Microbes, 5*(6), 696-710. doi:10.4161/19490976.2014.983768

Menees, S., & Chey, W. (2018). The gut microbiome and irritable bowel syndrome. *F1000Res, 7*. doi:10.12688/f1000research.14592.1

Mentella, M. C., Scaldaferri, F., Pizzoferrato, M., Gasbarrini, A., & Miggiano, G. A. D. (2020). Nutrition, IBD and gut microbiota: A review. *Nutrients, 12*(4). doi:10.3390/nu12040944

Merrick, B., Allen, L., Masirah M Zain, N., Forbes, B., Shawcross, D. L., & Goldenberg, S. D. (2020). Regulation, risk and safety of faecal microbiota transplant. *Infect Prev Pract, 2*(3). doi:10.1016/j.infpip.2020.100069

Miller, C. P., Bohnhoff, M., & Rifkind, D. (1956). The effect of an antibiotic on the susceptibility of the mouse's intestinal tract to *Salmonella* infection. *Trans Am Clin Climatol Assoc, 68*, 51-55; discussion 55-58.

Miller, M. A., Hyland, M., Ofner-Agostini, M., Gourdeau, M., & Ishak, M. (2002). Morbidity, mortality, and healthcare burden of nosocomial *Clostridium difficile*-associated diarrhea in Canadian hospitals. *Infect Control Hosp Epidemiol, 23*(3), 137-140. doi:10.1086/502023

Mills, J. P., Rao, K., & Young, V. B. (2018). Probiotics for prevention of *Clostridium difficile* infection. *Curr Opin Gastroenterol, 34*(1), 3-10. doi:10.1097/mog.0000000000000410

Moehring, R. W., Lofgren, E. T., & Anderson, D. J. (2013). Impact of change to molecular testing for *Clostridium difficile* infection on healthcare facility-associated incidence rates. *Infect Control Hosp Epidemiol, 34*(10), 1055-1061. doi:10.1086/673144

Monot, M., Eckert, C., Lemire, A., Hamiot, A., Dubois, T., Tessier, C., . . . Dupuy, B. (2015). *Clostridium difficile*: New insights into the evolution of the pathogenicity locus. *Sci Rep, 5*, 15023. doi:10.1038/srep15023

Moore, N. (2021). *Transcriptomic analysis of Clostridioides difficile grown in a biologically relevant growth medium.* Doctor of Philosophy(Ph.D.). Ulster Univeristy, 242 p. https://pure.ulster.ac.uk/ws/portalfiles/portal/90404356/2021MooreNPhD.pdf

Mullish, B. H., Quraishi, M. N., Segal, J. P., McCune, V. L., Baxter, M., Marsden, G. L., . . . Williams, H. R. T. (2018). The use of faecal microbiota transplant as treatment for recurrent or refractory *Clostridium difficile* infection and other potential indications: joint British Society of Gastroenterology (BSG) and Healthcare Infection Society (HIS) guidelines. *Gut, 67*(11), 1920-1941. doi:10.1136/gutjnl-2018-316818

Na, X., & Kelly, C. (2011). Probiotics in C*lostridium difficile* infection. *J Clin Gastroenterol, 45 Suppl*(Suppl), S154-158. doi:10.1097/MCG.0b013e31822ec787

Nagao-Kitamoto, H., Leslie, J. L., Kitamoto, S., Jin, C., Thomsson, K. A., Gillilland, M. G., 3rd, . . . Kamada, N. (2020). Interleukin-22-mediated host glycosylation prevents *Clostridioides difficile* infection by modulating the metabolic activity of the gut microbiota. *Nat Med, 26*(4), 608-617. doi:10.1038/s41591-020-0764-0

Navarro, M. A., McClane, B. A., & Uzal, F. A. (2018). Mechanisms of Action and Cell Death Associated with *Clostridium perfringens* Toxins. *Toxins (Basel), 10*(5). doi:10.3390/toxins10050212

Nawrocki, K. L., Edwards, A. N., Daou, N., Bouillaut, L., & McBride, S. M. (2016). CodY-Dependent Regulation of Sporulation in *Clostridium difficile*. *J Bacteriol, 198*(15), 2113-2130. doi:10.1128/JB.00220-16

Nerandzic, M. M., & Donskey, C. J. (2011). Effect of ceftobiprole treatment on growth of and toxin production by *Clostridium difficile* in cecal contents of mice. *Antimicrob Agents Ch, 55*(5), 2174-2177. doi:10.1128/AAC.01612-10

Ng, S. C., Hart, A. L., Kamm, M. A., Stagg, A. J., & Knight, S. C. (2009). Mechanisms of action of probiotics: recent advances. *Inflamm Bowel Dis, 15*(2), 300-310. doi:10.1002/ibd.20602

Nwachuku, E., Shan, Y., Senthil-Kumar, P., Braun, T., Shadis, R., Kirton, O., & Vu, T. Q. (2020). Toxic *Clostridioides* (formerly *Clostridium*) *difficile* colitis: No longer a diarrhea associated infection. *Am J Surg*. doi:10.1016/j.amjsurg.2020.06.026

Ofori, E., Ramai, D., Dhawan, M., Mustafa, F., Gasperino, J., & Reddy, M. (2018). Community-acquired *Clostridium difficile*: epidemiology, ribotype, risk factors, hospital and intensive care unit outcomes, and current and emerging therapies. *J Hosp Infect, 99*(4), 436-442. doi:10.1016/j.jhin.2018.01.015

Ohtani, K., Hayashi, H., & Shimizu, T. (2002). The luxS gene is involved in cell-cell signalling for toxin production in *Clostridium perfringens*. *Mol Microbiol, 44*(1), 171-179. doi:10.1046/j.1365-2958.2002.02863.x

Orrell, K. E., Zhang, Z., Sugiman-Marangos, S. N., & Melnyk, R. A. (2017). *Clostridium difficile* toxins A and B: Receptors, pores, and translocation into cells. *Crit Rev Biochem Mol Biol, 52*(4), 461-473. doi:10.1080/10409238.2017.1325831

Owens, R. C., Jr., Donskey, C. J., Gaynes, R. P., Loo, V. G., & Muto, C. A. (2008). Antimicrobial-associated risk factors for *Clostridium difficile* infection. *Clin Infect Dis, 46 Suppl 1*, S19-31. doi:10.1086/521859

Papoutsakis, E. T. (2008). Engineering solventogenic clostridia. *Curr Opin Biotechnol, 19*(5), 420-429. doi:10.1016/j.copbio.2008.08.003

Parada Venegas, D., De la Fuente, M. K., Landskron, G., Gonzalez, M. J., Quera, R., Dijkstra, G., . . . Hermoso, M. A. (2019). Short chain fatty acids (SCFAs)-mediated gut epithelial and immune regulation and its relevance for inflammatory bowel diseases. *Front Immunol, 10*, 277. doi:10.3389/fimmu.2019.00277

Paramsothy, S., Kamm, M. A., Kaakoush, N. O., Walsh, A. J., van den Bogaerde, J., Samuel, D., . . . Borody, T. J. (2017). Multidonor intensive faecal microbiota transplantation for active ulcerative colitis: a randomised placebo-controlled trial. *The Lancet, 389*(10075), 1218-1228. doi:10.1016/s0140-6736(17)30182-4

Parassol, N., Freitas, M., Thoreux, K., Dalmasso, G., Bourdet-Sicard, R., & Rampal, P. (2005). *Lactobacillus casei* DN-114 001 inhibits the increase in paracellular permeability of enteropathogenic *Escherichia coli*-infected T84 cells. *Res Microbiol, 156*(2), 256-262. doi:10.1016/j.resmic.2004.09.013

Patel, M. R. (2011). *Charaterization of a putative quorum sensing system in Clostridium difficile.* Doctor of Philosophy (Ph.D.) (University of Nottingham), (13570) 146 p. http://eprints.nottingham.ac.uk/13570/1/555418.pdf

Pépin, J., Saheb, N., Coulombe, M. A., Alary, M. E., Corriveau, M. P., Authier, S., . . . Lanthier, L. (2005). Emergence of fluoroquinolones as the predominant risk factor for *Clostridium difficile*-associated diarrhea: a cohort study during an epidemic in Quebec. *Clin Infect Dis, 41*(9), 1254-1260. doi:10.1086/496986

Pepin, J., Valiquette, L., & Cossette, B. (2005). Mortality attributable to nosocomial *Clostridium difficile*-associated disease during an epidemic caused by a hypervirulent strain in Quebec. *CMAJ, 173*(9), 1037-1042. doi:doi.org/10.1503/cmaj.050978

Pereira, C. S., Thompson, J. A., & Xavier, K. B. (2013). AI-2-mediated signalling in bacteria. *FEMS Microbiol Rev, 37*(2), 156-181. doi:10.1111/j.1574-6976.2012.00345.x

Pothoulakis, C., Kelly, C. P., Joshi, M. A., Gao, N., O'Keane, C. J., Castagliuolo, I., & Lamont, J. T. (1993). *Saccharomyces boulardii* inhibits *Clostridium difficile* toxin A binding and enterotoxicity in rat ileum. *Gastroenterology, 104*(4), 1108-1115. doi:10.1016/0016-5085(93)90280-p

Pothoulakis C., B. L., Ely R., Faris B., Clark ME., Franzblau C., LaMont JT. (1986). Purification and properties of *Clostridium difficile* cytotoxin B. *J Biol Chem, 261*(3), 1316-1321.

Pulikkan, J., Mazumder, A., & Grace, T. (2019). Role of the gut microbiome in autism spectrum disorders. *Adv Exp Med Biol, 1118*, 253-269. doi:10.1007/978-3-030-05542-4_13

Pultz, N. J., & Donskey, C. J. (2005). Effect of antibiotic treatment on growth of and toxin production by *Clostridium difficile* in the cecal contents of mice. *Antimicrobial agents and chemotherapy, 49*(8), 3529-3532. doi:10.1128/AAC.49.8.3529-3532.2005

Qin, H.-L., Shen, T.-Y., Gao, Z.-G., Fan, X.-B., Hang, X.-M., Jiang, Y.-Q., & Zhang, H.-Z. (2005). Effect of lactobacillus on the gut microflora and barrier function of the rats with abdominal infection. *World J gastroentero, 11*(17), 2591-2596. doi:10.3748/wjg.v11.i17.2591

Rea, M. C., Sit, C. S., Clayton, E., O'Connor, P. M., Whittal, R. M., Zheng, J., . . . Hill, C. (2010). Thuricin CD, a posttranslationally modified bacteriocin with a narrow spectrum of activity against *Clostridium difficile*. *Proc Natl Acad Sci U S A, 107*(20), 9352-9357. doi:10.1073/pnas.0913554107

Reineke, J., Tenzer, S., Rupnik, M., Koschinski, A., Hasselmayer, O., Schrattenholz, A., . . . von Eichel-Streiber, C. (2007). Autocatalytic cleavage of *Clostridium difficile* toxin B. *Nature, 446*(7134), 415-419. doi:10.1038/nature05622

Remy, B., Mion, S., Plener, L., Elias, M., Chabriere, E., & Daude, D. (2018). Interference in bacterial *Quorum Sensing*: A biopharmaceutical perspective. *Front Pharmacol, 9*, 203. doi:10.3389/fphar.2018.00203

Rinninella, E., Raoul, P., Cintoni, M., Franceschi, F., Miggiano, G. A. D., Gasbarrini, A., & Mele, M. C. (2019). What is the healthy gut microbiota composition? A changing dcosystem across age, environment, diet, and diseases. *Microorganisms, 7*(1). doi:10.3390/microorganisms7010014

Robinson, C. J., & Young, V. B. (2010). Antibiotic administration alters the community structure of the gastrointestinal micobiota. *Gut microbes, 1*(4), 279-284. doi:10.4161/gmic.1.4.12614

Rocha, D., Castro, T. L. P., Aguiar, E., & Pacheco, L. G. C. (2020). Gene expression analysis in bacteria by RT-qPCR. *Methods Mol Biol, 2065*, 119-137. doi:10.1007/978-1-4939-9833-3_10

Rolfe, R. D., & Finegold, S. M. (1979). Purification and characterization of *Clostridium difficile* toxin. *Infect Immun, 25*(1), 191-201. doi:10.1128/iai.25.1.191-201.1979

Roux, A., Todd, D. A., Velazquez, J. V., Cech, N. B., & Sonenshein, A. L. (2014). CodY-mediated regulation of the *Staphylococcus aureus* Agr system integrates nutritional and population density signals. *J Bacteriol, 196*(6), 1184-1196. doi:10.1128/JB.00128-13

Rutherford, S. T., & Bassler, B. L. (2012). Bacterial quorum sensing: its role in virulence and possibilities for its control. *Cold Spring Harb Perspect Med, 2*(11). doi:10.1101/cshperspect.a012427

Schäffler, H., & Breitrück, A. (2018). *Clostridium difficile* – From Colonization to Infection. *Front Microbiol, 9*(646). doi:10.3389/fmicb.2018.00646

Schiavo, G., Poulain, B., Rossetto, O., Benfenati, F., Tauc, L., & Montecucco, C. (1992). Tetanus toxin is a zinc protein and its inhibition of neurotransmitter release and protease activity depend on zinc. *EMBO J, 11*(10), 3577-3583.

Schwan, C., Stecher, B., Tzivelekidis, T., van Ham, M., Rohde, M., Hardt, W. D., . . . Aktories, K. (2009). *Clostridium difficile* toxin CDT induces formation of microtubule-based protrusions and increases adherence of bacteria. *PLoS Pathog, 5*(10), e1000626. doi:10.1371/journal.ppat.1000626

Seekatz, A. M., Theriot, C. M., Rao, K., Chang, Y. M., Freeman, A. E., Kao, J. Y., & Young, V. B. (2018). Restoration of short chain fatty acid and bile acid metabolism following fecal microbiota transplantation in patients with recurrent *Clostridium difficile* infection. *Anaerobe, 53*, 64-73. doi:10.1016/j.anaerobe.2018.04.001

Shrestha, A., Uzal, F. A., & McClane, B. A. (2018). Enterotoxic clostridia: *Clostridium perfringens* enteric diseases. *Microbiol Spectr, 6*(5). doi:10.1128/microbiolspec.GPP3-0003-2017

Simor, A. E. (2010). Diagnosis, management, and prevention of *Clostridium difficile* infection in long-term care facilities: a review. *J Am Geriatr Soc, 58*(8), 1556-1564. doi:10.1111/j.1532-5415.2010.02958.x

Simor, A. E., Bradley, S. F., Strausbaugh, L. J., Crossley, K., Nicolle, L. E., & Committee, S. L.-T.-C. (2002). *Clostridium difficile* in long-term-care facilities for the elderly. *Infect Control Hosp Epidemiol, 23*(11), 696-703. doi:10.1086/501997

Slater, R. T., Frost, L. R., Jossi, S. E., Millard, A. D., & Unnikrishnan, M. (2019). *Clostridioides difficile* LuxS mediates inter-bacterial interactions within biofilms. *Sci Rep, 9*(1), 9903. doi:10.1038/s41598-019-46143-6

Smits, W. K., Lyras, D., Lacy, D. B., Wilcox, M. H., & Kuijper, E. J. (2016). *Clostridium difficile* infection. *Nat Rev Dis Primers, 2*, 16020. doi:10.1038/nrdp.2016.20

Sniffen, J. C., McFarland, L. V., Evans, C. T., & Goldstein, E. J. C. (2018). Choosing an appropriate probiotic product for your patient: An evidence-based practical guide. *PLoS One, 13*(12), e0209205. doi:10.1371/journal.pone.0209205

Song, J. H., & Kim, Y. S. (2019). Recurrent *Clostridium difficile* infection: risk factors, treatment, and prevention. *Gut Liver, 13*(1), 16-24. doi:10.5009/gnl18071

Speri, E., Janardhanan, J., Masitas, C., Schroeder, A., Lastochkin, E., Wolter, W. R., . . . Chang, M. (2020). Discovery of a potent picolinamide antibacterial active against *Clostridioides difficile*. *ACS Infect Dis, 6*(9), 2362-2368. doi: 10.1021/acsinfecdis.0c00479.

Stabler, R. A., He, M., Dawson, L., Martin, M., Valiente, E., Corton, C., . . . Wren, B. W. (2009). Comparative genome and phenotypic analysis of *Clostridium difficile* 027 strains provides insight into the evolution of a hypervirulent bacterium. *Genome Biol, 10*(9), R102. doi:10.1186/gb-2009-10-9-r102

Tabasco, R., Garcia-Cayuela, T., Pelaez, C., & Requena, T. (2009). *Lactobacillus acidophilus* La-5 increases lactacin B production when it senses live target bacteria. *Int J Food Microbiol, 132*(2-3), 109-116. doi:10.1016/j.ijfoodmicro.2009.04.004

Tariq, R., Saha, S., Solanky, D., Pardi, D. S., & Khanna, S. (2020). Predictors and management of failed fecal microbiota transplantation for recurrent *Clostridioides difficile* Infection. *J Clin Gastroenterol*. doi:10.1097/MCG.0000000000001398

Taylor, N. S., Thorne, G. M., & Bartlett, J. G. (1981). Comparison of two toxins produced by *Clostridium difficile*. *Infect Immun, 34*(3), 1036-1043. doi:10.1128/IAI.34.3.1036-1043.1981

Theriot, C. M., Bowman, A. A., & Young, V. B. (2016). Antibiotic-induced alterations of the gut microbiota alter secondary bile acid production and allow for *Clostridium difficile* spore germination and outgrowth in the large intestine. *mSphere, 1*(1). doi:10.1128/mSphere.00045-15

Theriot, C. M., Koenigsknecht, M. J., Carlson, P. E., Jr., Hatton, G. E., Nelson, A. M., Li, B., . . . Young, V. B. (2014). Antibiotic-induced shifts in the mouse gut microbiome and metabolome increase susceptibility to *Clostridium difficile* infection. *Nat Commun, 5*, 3114. doi:10.1038/ncomms4114

Vonaesch, P., Anderson, M., & Sansonetti, P. J. (2018). Pathogens, microbiome and the host: emergence of the ecological Koch's postulates. *FEMS Microbiol Rev, 42*(3), 273-292. doi:10.1093/femsre/fuy003

Warny, M., Pepin, J., Fang, A., Killgore, G., Thompson, A., Brazier, J., . . . McDonald, L. C. (2005). Toxin production by an emerging strain of *Clostridium difficile* associated with outbreaks of severe disease in North America and Europe. *The Lancet, 366*(9491), 1079-1084. doi:10.1016/s0140-6736(05)67420-x

Wedel, N., Toselli, P., Pothoulakis, C., Faris, B., Oliver, P., Franzblau, C., & Lamont, T. (1983). Ultrastructural effects of *Clostridium difficile* toxin B on smooth muscle cells and fibroblasts. *Exp Cell Resh, 148*(2), 413-422. doi:https://doi.org/10.1016/0014-4827(83)90163-5

Winzer, K., & Hardie, W. P. (2003). LuxS and autoinducer-2: their contribution to quorum. *Adv Appl Microbiol, 53*, 291.

Yan, F., Cao, H., Cover, T. L., Whitehead, R., Washington, M. K., & Polk, D. B. (2007). Soluble proteins produced by probiotic bacteria regulate intestinal epithelial cell survival and growth. *Gastroenterology, 132*(2), 562-575. doi:10.1053/j.gastro.2006.11.022

Yong, C. C., Lim, J., Kim, B. K., Park, D. J., & Oh, S. (2019). Suppressive effect of *Lactobacillus fermentum* Lim2 on *Clostridioides difficile* 027 toxin production. *Lett Appl Microbiol, 68*(5), 386-393. doi:10.1111/lam.13124

Yun, B., Oh, S., & Griffiths, M. W. (2014). *Lactobacillus acidophilus* modulates the virulence of *Clostridium difficile*. *J Dairy Sci, 97*(8), 4745-4758. doi:10.3168/jds.2014-7921

APPENDIX

Table 6. Bioanalysis of RNA extraction from *C. difficile* R20291 reference cultures and co-cultures with microbiota.

Experiment ID	Sample ID	RNA concentration (ng/µL)	Ratio 260/280	Ratio 260/230
Repetition 1	REF 1 T1	138.0	2.04	1.78
	REF 1 T3	380.0	2.06	1.98
	REF 1 T5	326.7	2.06	2.02
	CTRL 1 T1	483,7	2.01	1.90
	CTRL 1 T3	475.8	2.02	1.90
	CTRL 1 T5	339.9	2.02	1.82
	ATB 1 T1	134.1	2.00	1.74
	ATB 1 T3	173.1	1.88	1.59
	ATB 1 T5	113.2	1.82	1.56
	PRO 1 T1	122.9	1.98	1.66
	PRO 1 T3	176.9	1.98	1.79
	PRO 1 T5	123.3	1.91	1.57
	COMBIN 1 T1	181.9	1.99	1.52
	COMBIN 1 T3	257.3	2.02	1.78
	COMBIN 1 T5	164.7	1.96	1.63
Repetition 2	REF 2 T1	344.3	2.05	2.04
	REF 2 T3	374.8	2.04	2.01
	REF 2 T5	323.2	2.05	2.08
	CTRL 2 T1	178.9	1.94	1.68
	CTRL 2 T3	166.4	1.91	1.55
	CTRL 2 T5	181.3	1.93	1.65
	ATB 2 T1	464.5	1.88	1.84
	ATB 2 T3	509.6	1.94	1.94
	ATB 2 T5	407.0	1.79	1.80
	PRO 2 T1	420.3	2.02	2.02
	PRO 2 T3	410.6	2.02	2.04
	PRO 2 T5	320.6	1.94	1.76
	COMBIN 2 T1	507.1	1.88	1.79
	COMBIN 2 T3	571.8	1.86	1.95
	COMBIN 2 T5	289.9	1.80	1.57
Repetition 3	REF 3 T1	94.9	2.00	1.59
	REF 3 T3	179.8	2.06	1.80
	REF 3 T5	74.2	1.95	1.25
	CTRL 3 T1	337.5	1.99	1.80
	CTRL 3 T3	239.6	1.99	1.74

Repetition 3 (suite)	CTRL 3 T5	206.5	1.98	1.71
	ATB 3 T1	282.5	1.90	1.66
	ATB 3 T3	190.8	1.90	1.63
	ATB 3 T5	228.7	1.88	1.73
	PRO 3 T1	167.8	1.95	1.52
	PRO 3 T3	184.0	1.92	1.63
	PRO 3 T5	146.3	1.88	1.54
	COMBIN 3 T1	210.8	1.98	1.70
	COMBIN 3 T3	290.3	2.03	1.84
	COMBIN 3 T5	178.2	2.00	1.77

SOMMAIRE RÉCAPITULATIF EN FRANÇAIS

1. INTRODUCTION

1.1 *Clostridioides difficile*

Le genre *Clostridium* est un des plus larges genres chez les procaryotes et contient des bactéries à Gram positif, en forme de bâtonnet et sporulantes (Cruz-Morales et al., 2019). Certaines espèces pathogéniques du genre *Clostridium* sont responsables de plusieurs problèmes de santé majeurs chez les humains et les animaux, le plus souvent suite à la relâche de toxines comme des neurotoxines par *Clostridium botulinum* (A. T. Carter & Peck, 2015*)* et *Clostridium tetani* (Chapeton-Montes et al., 2020; Schiavo et al., 1992*)* et des entéro- ou cyto-toxines par *Clostridium perfringens* (Navarro, McClane, & Uzal, 2018; Shrestha, Uzal, & McClane, 2018) et *Clostridioides difficile* (Chandrasekaran & Lacy, 2017). *C.difficile* (autrefois connu comme *Clostridium difficile*) est une bactérie pathogène opportuniste qui est mieux connue comme étant l'agent causal des infections à *C. difficile* (ICD) se présentant normalement par une diarrhée associée à *C. difficile* (DACD) ou des colites (CACD). Le pathogène, initialement appelé *Bacillus difficilis,* a été découvert et caractérisé pour la première fois par Hall et Tool en 1935. Toutefois, son association aux diarrhées associées aux antibiotiques (DAA) a été documenté seulement en 1978 (George et al., 1978; Larson, Price, Honour, & Borriello, 1978). De nos jours, *C. difficile* est reconnu comme un des pathogènes nosocomiaux les plus importants.

1.2 Infections à *C. difficile* (ICD)

Les ICD peuvent se développer suite à l'ingestion de spores de *C. difficile* qui sont transmises par la route fécale-orale. Les spores proviennent de multiples sources biologiques comme les porteurs asymptomatiques, les patients infectés et le tractus gastro-intestinal (TGI) des animaux, mais également d'environnements contaminés où les spores peuvent reposer sur des surfaces rigides et être dispersées par le personnel clinique et les patients, ce qui représente un problème majeur en milieu hospitalier. Les spores sont résistantes à la chaleur, aux antibiotiques et à la majorité des désinfectants courants. De plus, puisque les spores ne sont pas affectées par le pH acide de l'estomac, elles poursuivent leur route à travers le TGI et atteingnent les intestins. La germination des spores est ensuite facilitée par une alteration des ratios des acides biliaires primaires et secondaires, ces derniers agissant comme germinants dans le petit intestin. Les cellules

végétatives maintenant métaboliquement actives vont ensuite coloniser l'hôte en occupant des niches vacantes dans l'intestin distal, le plus souvent après la perturbation du microbiote suite à des traitements antibiotiques et vont amorcer la production des toxines A et B, ce qui va mener à la diarrhée (Figure 1) (Hookman & Barkin, 2009). Les ICD représentent environ le tiers des infections associées aux antibiotiques (Magill et al., 2014; Simor, 2010).

Les ICD causent habituellement des diarrhées aqueuses qui apparaissent au bout de 2 à 3 jours, mais celles-ci peuvent apparaître plus tard, même après quelques mois, dépendamment de l'administration d'agents antimicrobiens et même être absentes dans certains cas (Nwachuku et al., 2020). La sévérité des ICD est relativement large avec des symptômes allant de légers à sévères, incluant le plus souvent de la diarrhée et des colites, mais également des colites pseudomembraneuses, le mégacolon toxique, la septicémie et même la mort. Généralement, les fèces molles, la distension abdominale et/ou une leucocytose font partie du diagnostique précoce (Hookman & Barkin, 2009). Les ICD récurrentes (ICDr) ne sont pas rares puisque 10-30% des patients infectés une première fois vont rechuter une seconde fois et les incidences de rechutes subséquentes peuvent atteindre 45-65%. Les ICDr sont généralement associées avec un séjour prolongé en milieu hospitalier, l'usage d'antibiotiques, l'âge au-delà de 65 ans, des co-morbidités et la médication anti-acide (Finn, Andersson, & Madin-Warburton, 2021; Song & Kim, 2019).

En plus de causer la mort, la gestion et le traitement des ICD sont très couteux et représentent une lourde charge financière pour les établissements hospitaliers et les gouvernements (Dubberke, Reske, Olsen, McDonald, & Fraser, 2008). En ce qui a trait uniquement aux DACD, il a été estimé que les réadmissions coûtent un minimum de 128 200 $ anuellement par établissement au Canada (M. A. Miller, Hyland, Ofner-Agostini, Gourdeau, & Ishak, 2002).

1.2.1 Épidémiologie

Les ICD représentent un problème mondial majeur dû à de multiples souches épidémiques activement présentes en Amérique du Nord, en Europe, au Moyen-Orient et en Asie. Depuis 2005, la souche dénommée NAP1, pour *North American pulsed-field* 1, ou ribotype PCR 027 (RT 027), est fréquemment détectée chez les patients infectés en Amérique du Nord et en Europe. En plus de démontrer une nouvelle résistance aux fluoroquinolones, cette souche produit une troisième toxine, la toxine binaire, et possède un gène *tcdC* muté, ce qui résulte en une production accrue des toxines A et B. Ces caractéristiques accentuent la virulence de la souche NAP1, ce qui mène à son association avec des taux accrus de morbidité et de récurrence (MacCannell et al., 2006; Pépin et

al., 2005; Pepin, Valiquette, & Cossette, 2005; Warny et al., 2005). En 2011, *C. difficile* était l'agent responsable de plus de 450 000 infections et était associé avec approximativement 29 000 morts aux États-Unis (Lessa et al., 2015). Dans le groupe d'âge de 65 ans et plus à lui seul, l'incidence s'élevait à 627,7 par 100 000 personnes, ce qui était 4 fois plus élevé que l'incidence dans le groupe d'âge de 45-65 ans et presque 9 fois plus élevé que l'incidence chez les personnes âgées de 44 ans et moins (Lessa et al., 2015). La souche NAP1 a également été associée avec un nombre élevé de cas acquis en communauté. Les dernières mises à jour montrent une importante augmentation dans les ICD acquises en communauté, allant de 170 000 cas en 2011 à 226 400 cas en 2017 aux États-Unis (Guh et al., 2020).

Malgré que les hauts taux de mortalité associés aux ICD demeurent un problème majeur encore aujourd'hui, la sensibilitation entourant l'usage des antibiotiques à large spectre et d'autres initiatives comme une meilleure gestion des antibiotiques (*antibiotic stewardship*), semblent avoir prouvées leur efficacité dans le contrôle de la maladie suite aux dernières éclosions. Dans un récent rapport par le *Canadian Nosocomial Infection Surveillance Program*, il est mentionné que le taux national d'ICD nosocomiales a démontré une diminution relative de 35,8% en 2015. Également, la prévalence de la souche NAP1 (RT 027) a diminué tandis que celles des souches NAP4 et NAP11 ont augmentée. Ces souches étaient prévalentes à 37.6%, 14.2% et 5.9% respectivement et représentaient plus de la moitié des 2690 isolats testés durant cette période (Katz et al., 2018). Les plus récentes données publiées par le *Centers for Disease Control and Prevention* (CDC) rapportaient que le nombre d'ICD nosocomiales ainsi que les hospitalisations aux États-Unis avaient diminué de 36% et 25% respectivement de 2011 à 2017. Similaire à la tendance observée au Canada, le ribotype 027 a diminué de manière significative durant cette période. Il a été proposé que la réduction de l'usage des fluroquinolones aurait contribué au déclin de cette souche (Guh et al., 2020).

1.2.2 Facteurs de risque

Les facteurs de risque clés associés au développement des ICD incluent l'exposition aux antibiotiques, l'âge avancé (> 65 ans) et l'hospitalisation (Czepiel et al., 2019; Pepin et al., 2005). La plupart des cas d'ICD surviennent chez les patients âgés, ce qui est démontré par une augmentation du risque d'ICD de 5-10 fois chez les patients âgés de 65 ans et plus. Aussi, l'âge avancé represente un risque significatif au développement de complications ainsi que d'être associé à des sévérité et mortalité accrues (Czepiel et al., 2019; Leffler & Lamont, 2015). L'incidence de la colonisation à *C. difficile* (symptomatique ou non) dès les premières journées d'hospitalisation

varie entre 2.1-20.0% et est corrélée positivement avec la durée de l'hébergement en milieu hospitalier, où l'incidence peut atteindre plus de 50% après 1 mois ou moins d'hospitalisation (Czepiel et al., 2019). La prise d'antibiotiques est considérée comme le plus important facteur de risque pour le développement des DACD (Hookman & Barkin, 2009). Les personnes en santé et immunocompétentes sont généralement non permissifs à la colonisation de *C. difficile* puisque le pathogène échoue à proliférer de manière efficace dans le TGI, et est donc incapable d'exprimer ses facteurs de virulence comme ses toxines (Lawley & Walker, 2013). L'administration d'antibiotiques représente le plus grand risque pour le développement des ICD puisqu'elle a été associée avec une réduction drastique des niveaux de diversité bactérienne et avec d'importants changements dans la composition des taxa (Lagier, 2016; Robinson & Young, 2010; Theriot et al., 2014), ce qui facilite la prolifération de *C. difficile*. L'administration d'antibiotiques a en effet résulté en l'altération des microbiotes intestinaux chez une variété de modèles animaux (Best, Freeman, & Wilcox, 2012; Chen et al., 2008; Robinson & Young, 2010; Theriot et al., 2014) et chez les humains (Ghimire et al., 2020). Les antibiotiques les plus fréquemment impliqués dans les ICD incluent la clindamycine, les céphalosporines et les fluoroquinolones (Gerding, 2004; Lagier, 2016; Owens, Donskey, Gaynes, Loo, & Muto, 2008; Schäffler & Breitrück, 2018).

1.2.3 Traitements

Malgré le fait que les antibiotiques sont fortement associés avec le développement de l'infection, ces derniers sont encore couramment utilisés pour traiter les ICD. Récemment, la fidaxomicine a été reconnue comme traitement préféré pour les épisodes initiaux d'ICD, mais les antibiotiques comme la vancomycine et le métronidazole (pour les ICD non sévères) demeurent des alternatives acceptables compte tenu de leur plus faible coût et de leur plus grande disponibilité comparativement à la fidaxomicine. Les cas sévères ou fulminants sont traités avec des doses supérieures de ces mêmes antibiotiques, une combinaison d'antibiotiques et/ou par l'utilisation de routes d'administration alternatives (*i.e.* via un tube nasogastrique, par voie rectale ou par voie intraveineuse). En plus de l'antibiothérapie traditionnelle, la thérapie de transplantation de microbiote fécal (TMF), a été incluse comme traitement des épisodes récurrents à partir de la 2^{e} récurrence (Johnson et al., 2021; Kukla et al., 2020).

1.3 Facteurs de virulence de *C. difficile*

Les toxines A et B de *C. difficile*, sont reconnues comme la principale cause de la manifestation de l'ICD chez les patients infectés. Toutefois, d'autres facteurs de virulence comme la sporulation et la mobilité contribuent aussi à la virulence de la bactérie. En effet, la capacité de la bactérie de former des spores promeut sa dispersion dans l'environnement hospitalier, contribuant à sa transmission. Finalement, la mobilité, l'adhésion et la résistance aux antibiotiques contribuent à l'expansion de *C. difficile* dans le système digestif suite à la perturbation du microbiote par les antibiotiques (Awad, Johanesen, Carter, Rose, & Lyras, 2014).

1.3.1 Toxines de *C. difficile*

La toxine A (TcdA) et la toxine B (TcdB), dont les gènes sont localisés sur un locus de pathogénécité (PaLoc) de 19.6 kb, sont responsables des symptômes typiques de l'ICD (Awad et al., 2014; Smits, Lyras, Lacy, Wilcox, & Kuijper, 2016). Les gènes des toxines sont encadrés de trois gènes accessoires soient *tcdC* et *tcdR* qui sont impliqués dans leur régulation et *tcdE,* impliqué dans leur sécrétion. Une troisième toxine, la toxine binaire (CDT) a été identifiée dans 23% des souches virulentes comme les ribotypes 027 et 078 (Bauer et al., 2011). Il a été démontré que la toxine CDT accentue la virulence de ces souches (Cowardin et al., 2016; Schwan et al., 2009). La toxine CDT est une actine-ADP-ribosyltransférase et est codée par les gènes *cdtA* et *cdtB* localisés sur un second locus nommé le locus Cdt ou « CdtLoc » (Figure 2).

Suite à l'expansion de *C. difficile* dans le TGI et la relâche des toxines, TcdA et TcdB entrent dans les cellules épithéliales par endocytose. Suite à l'acidification des vésicules de l'endosome et la formation d'un pore, les toxines sont transférées dans le cytosol où le domaine glucosyltransférase (GTD) des toxines est clivé et relâché (Orrell, Zhang, Sugiman-Marangos, & Melnyk, 2017; Reineke et al., 2007). L'activité glucosyltransférase des toxines inactive les GTPases Rho et Ras de l'hôte via glycolysation ce qui mène à la perturbation des fonctions des cellules de l'hôte (Chandrasekaran & Lacy, 2017). Les deux toxines démontrent des effets cytotoxiques sur une variété de types cellulaires *in vitro* (Pothoulakis C., 1986; Taylor, Thorne, & Bartlett, 1981; Wedel et al., 1983). Tel que documenté par Chandrasekeran et Lacy (2017), l'entrée des toxines dans les cellules épithéliales du colon mène à des effets cytopathiques et cytotoxiques qui incluent la mort cellulaire, la perturbation des jonctions serrées et de l'intégrité épithéliale, une augmentation de la perméabilité de la muqueuse et de la sécrétion de fluide, ainsi qu'une réponse inflammatoire aïgue via l'induction de la production de cytokines pro-inflammatoires, le recruitement des neutrophiles et le dommage tissulaire.

1.4 L'implication du *Quorum sensing* dans la virulence des pathogènes

Le *quorum sensing* (QS) est une forme de communication intercellulaire possible via la relâche et la réception de molécules apellées auto-inducteurs (AI) par les bactéries. Au fur et à mesure que la population grossit, les AI s'accumulent dans l'environnement. Ainsi, cette communication permet à une communauté bactérienne de coordonner certains processus cellulaires en fonction de la densité de la population (de Kievit & Iglewski, 2000). Le QS a été associé à la régulation de facteurs de virulence comme la sporulation, la formation de biofilms et la production de toxines, chez de nombreuses bactéries pathogènes. Trois systèmes ont été décrits jusqu'à maintenant, dont deux qui représentent une communication intra-espèce et qui diffèrent entre les bactéries à Gram négatif et Gram positif. Chez une majorité des bactéries à Gram négatif, les auto-inducteurs consistent en de petites molécules diffusibles, soient les *N*-acyl homosérine lactones (AHL) (de Kievit & Iglewski, 2000). Les systèmes de QS *las* et *rhl* chez *Pseudomonas aeruginosa,* un pathogène opportuniste, font partie des systèmes de QS les mieux connus pour leur implication importante dans la pathogénicité, ce qui a d'ailleurs été démontré dans nombreux modèles animaux (Deep, Chaudhary, & Gupta, 2011). Le système de QS chez les bactéries à Gram positif est discuté plus loin. Finalement, le système LuxS/AI-2 a été découvert dans presque toutes les bactéries testées et est présent chez les bactéries à Gram négatif et Gram positif. Dûe à sa faible spécificité, il a été suggéré que ce système sert de communication inter-espèces (Bassler, Greenberg, & Stevens, 1997). Ce système implique un enzyme du métabolisme de la méthionine, LuxS. Cet enzyme produit le 4,5-dihydroxy-2,3-pentadione (DPD), le précurseur de la molécule signal furanosyl borate diester (AI-2) (Winzer & Hardie, 2003). Il a été démontré que ce système est impliqué dans la virulence, notamment quant à la production de biofilms, la motilité et la production de toxines (Elvers & Park, 2002; Girón, Torres, Freer, & Kaper, 2002; Hammer & Bassler, 2003; Ohtani, Hayashi, & Shimizu, 2002).

1.4.1 Le système de QS chez les bactéries à Gram positif

Chez les bactéries à Gram positif, un peptide est utilisé comme molécule signal. Généralement, le peptide précurseur est simultanément modifié et transporté à l'extérieur de la cellule via une protéine transmembranaire. Le peptide mature relâché dans l'environnement peut ensuite lier une protéine histidine kinase à la surface de la cellule, ce qui engendre l'autophosphorylation de cette dernière. Une cascade de phosphorylation mène ensuite à l'activation d'un régulateur ce qui engendre la modulation de gènes cibles (de Kievit & Iglewski, 2000). Le système de QS de *Staphylococcus aureus,* le système *agr* (« *accessory gene regulator* »)

est parmi l'un des mieux compris chez les bactéries à Gram positif. Le système *agr* comprend deux opérons, RNAII et RNAIII (Figure 3). Le premier contient quatre gènes nommés *agrBDAC.* AgrB et AgrD sont responsables de produire et de libérer le signal respectivement. AgrC et AgrA codent pour les deux composantes du système de transduction du signal, soient le récepteur kinase et le régulateur de la famille des régulateurs LytTR respectivement. AgrA lie les promoteurs P2 et P3, ce qui permet d'activer la transcription de son propre opéron (RNAII) mais également, la transcription de l'ARN régulateur RNAIII, impliqué dans l'activation de la production de facteurs de virulence. Le système *agr* est d'ailleurs impliqué dans la transition d'un état de cellules dormantes (biofilm) vers un état de cellules planctoniques et d'infection active et via la régulation positive de facteurs de virulence comme l'α-toxine (Rutherford & Bassler, 2012).

Autrement, il a été démontré que le système *agr* est impliqué dans la virulence chez plusieurs membres pathogènes du genre *Clostridium* tels que *C. botulinum* (Cooksley et al., 2010)*, C. perfringens* (J. Chen, Rood, & McClane, 2011) et *C. difficile* (Darkoh, DuPont, Norris, & Kaplan, 2015; M. J. Martin et al., 2013).

1.4.2 Régulation de la virulence par le système *agr* chez *C. difficile*

Des homologues des gènes *agr* ont été découverts dans le génome de la souche toxigénique *C. difficile* R20291 (RT 027) sur deux locus distincts soient, le locus *agr1* qui inclue des gènes fonctionnels *agrB1* et *agrD1* et le locus *agr2* qui inclue les gènes *agrA2, agrC2, agrB2* et *agrD2* (M. J. Martin et al., 2013; Stabler et al., 2009). Toutefois, dans certaines souches toxigéniques telle que *C. difficile* 630, seul le locus *agr* partiel contenant seulement *agrB* et *agrD* a été identifié. Le locus *agr* complet retrouvé chez *C. difficile* R20291 est semblable au locus de *S. aureus* mais l'ordre des gènes est inversé (Figure 4). AgrA, AgrC et AgrB partagent 28, 23 et 25% d'homologie en acides aminés avec les protéines de *S. aureus* respectivement. Cependant, AgrD, le précurseur du AIP, ne présente pas de similitude avec *S. aureus* (Melissa J. Martin et al., 2013).

Chez *C. difficile,* il a été démontré qu'AgrA est impliqué dans la régulation de *tcdA* mais aussi dans la régulation de gènes impliqués dans la biosynthèse flagellaire, dans les systèmes à deux composantes et dans la régulation du c-di-GMP (Melissa J. Martin et al., 2013). En effet, Martin *et al.* (2013) ont observé la répression de *tcdA* dans un mutant *agrA.* Ce mutant présentait également une diminution de la production de la toxine A (Melissa J. Martin et al., 2013).

Quant à *agrBD,* leur rôle dans la pathogénèse de *C. difficile* a été caractérisé via l'utilisation de mutants pour les gènes *agrB* et *agrD* des locus 1 (*i.e.* le locus partiel : *argB1D1*) et locus 2 (*i.e.*

le locus complet : *agrA2C2B2D2*) (Darkoh, Odo, & DuPont, 2016). Ce groupe a démontré que, contrairement au locus 2, le locus *agr1* est essentiel à la virulence de *C. difficile,* compte tenu que le mutant Δ*agrB1D1* démontre une absence de transcription de *tcdA* et *tcdB* ainsi qu'une activité abolie des toxines. Également, malgré que ce mutant soit capable de coloniser les souris, aucune toxine n'a pu être détectée dans les matières fécales. Le taux de survie pour les souris infectées avec le mutant était de 92% contrairement au taux de survie de 8% lorsque les souris étaient infectées avec la souche sauvage. À l'inverse, le mutant Δ*agrB2D2* conservait une virulence semblable à la souche sauvage.

1.4.3 Régulation de la virulence par le système LuxS/AI-2 chez *C. difficile*

L'implication du système LuxS/AI-2 dans la virulence de *C. difficile* demeure controversée. Certaines études ont conclu que ce système joue bel et bien un rôle dans la modulation de certains facteurs de virulence comme les gènes impliqués dans la production de toxines et la formation de biofilm (Đapa et al., 2013; A. S. Lee & Song, 2005; Slater et al., 2019). Des opinions oppposées suggèrent plutôt que le AI-2 ne représente qu'un produit dérivé du cycle de la méthionie et que son transport à l'extérieur de la cellule soit lié à sa toxicité potentielle (Glen et al., 2005). Ces derniers ont notamment démontré que le AI-2 n'était pas impliqué dans la régulation de la production des toxines. Quelques années plus tard, Patel (2011) a observé que le surnageant d'un mutant *luxS* démontrait une cytotoxicité envers les cellules Vero et HT29 semblable à la cytotoxicité démontrée par la souche sauvage, en plus d'observer que l'inactivation de *luxS* n'affectait ni la sporulation ni la germination (Patel, 2011). Ces observations supporteraient l'idée que le AI-2 ne serait pas directement impliqué dans la régulation des toxines chez *C. difficile.* Autrement, malgré que le rôle du système LuxS/AI-2 dans la virulence chez *C. difficile* ne soit toujours pas élucidé, ce système est bel et bien impliqué dans la virulence d'une variété de pathogènes, comme il a été documenté par Pereira, Thompson, et Xavier (2013).

1.5 La capacité du microbiote intestinal à résister à l'invasion des pathogènes

1.5.1 Le concept de résistance à la colonisation

Dans le microbiote du colon, qui est le plus large réservoir de microorganismes chez les mammifères, on retrouve cinq phyla primordiaux soient les *Firmicutes* et *Bacteroidetes* qui représentent près de 90% du microbiote, suivi des *Actinobacteria, Proteobacteria* et *Verrucomicrobia* (Arumugam et al., 2011; Backhed, Ley, Sonnenburg, Peterson, & Gordon, 2005; Rinninella et al., 2019). Le microbiote consite en un large écosystème équilibré qui joue un rôle

primordial dans le développement et la physiologie chez l'hôte, mais également dans la protection contre l'invasion des pathogènes, qui est désignée comme la résistance à la colonisation. Celle-ci fait interagir entre eux les pathogènes envahissants, le microbiote ainsi que le système immunitaire de l'hôte via divers mécanismes (Lawley & Walker, 2013). Parmis ces mécanismes, on retrouve l'exclusion compétitive via la disponibilité des niches de nutriments (Girinathan et al., 2020; Jenior ML, 2017; Litvak & Baumler, 2019), l'exclusion par la production d'acides gras à chaînes courtes (AGCCs) (Fachi et al., 2019; Louis & Flint, 2017; Parada Venegas et al., 2019), le métabolisme des acides biliaires (Nagao-Kitamoto et al., 2020; Seekatz et al., 2018; Theriot, Bowman, & Young, 2016) et la production de molécules inhibitrices (Khattab, Ahmed, Ragab, & Rasmy, 2020; Rea et al., 2010). Finalement, la perte de la résistance à la colonisation à été associée avec la perturbation du microbiote intestinal, aussi nommée « dysbiose intestinale ».

1.5.2 La dysbiose intestinale

La dysbiose intestinale est définie par un déséquilibre du microbiote (Hooks & O'Malley, 2017). Par ailleurs, ce déséquilibre - ou perte de l'homéostasie -, peut être caractérisé par une diversité du microbiote réduite, une couche de mucus amincie ainsi que des dommages à la muqueuse épithéliale et une inflammation accrue (Vonaesch, Anderson, & Sansonetti, 2018). La dysbiose intestinale est de plus en plus reconnue comme agent causal dans de nombreuses maladies intestinales. En effet, la dysbiose intestinale a été associée à une sévérité accrue dans de multiples maladies intestinales incluant le syndrôme du colon irritable (SCI), le cancer colorectal et le diabète de type 2, et serait aussi impliquée dans l'obésité et l'autisme (Amabebe, Robert, Agbalalah, & Orubu, 2020; Ebrahimzadeh Leylabadlo, Sanaie, Sadeghpour Heravi, Ahmadian, & Ghotaslou, 2020; Gagniere et al., 2016; Menees & Chey, 2018; Mentella, Scaldaferri, Pizzoferrato, Gasbarrini, & Miggiano, 2020; Pulikkan, Mazumder, & Grace, 2019).

Le microbiote intestinal en dysbiose a été associé avec la perte de la résistance à la colonisation pour la première fois en 1956 où les investigateurs ont démontré que la dose infectieuse de *Salmonella enterica* serovar Typhimurium était de 100 000 fois réduite si un traitement antibiotique précédait l'infection chez les souris (C. P. Miller, Bohnhoff, & Rifkind, 1956). Le concept de perte de la résistance à la colonisation suite à la perturbation du microbiote intestinal par les antibiotiques a par la suite été investigué dans les infections à *C. difficile.* Il a été démontré dans plusieurs études que la dysbiose induite par les antibiotiques était nécessaire pour permettre la prolifération et la production de toxines par *C. difficile* (Borriello & Barclay, 1986; Borriello, Barclay, & Welch, 1988; Nerandzic & Donskey, 2011; Pultz & Donskey, 2005).

Par ailleurs, la capacité intrinsèque de résistance à la colonisation du microbiote intestinal est un concept majeur ayant guidé le développement de thérapies alternatives aux antibiotiques pour la prévention de la colonisation des pathogènes et les infections.

1.6 Thérapies alternatives pour la prévention et le traitement des ICD

La rapide accumulation d'épidémies causées par la souche NAP1 a été associée avec sa résistance accrue aux antibiotiques. En effet, cette souche démontre une susceptibilité réduite aux antibiotiques tels que la rifampicine, la clindamycine, le chloramphénicol, la moxifloxacine et l'imipenem (Krutova et al., 2018). Malgré cette multi-résistance aux antibiotiques, ces derniers sont encore aujourd'hui utilisés pour le traitement des ICD. Il existe ainsi un besoin critique de trouver des thérapies alternatives aux antibiotiques pour traiter et prévenir les ICDs.

Parmi ces alternatives, on retrouve la transplantation de microbiote fécal (TMF), les vaccins (A. D. Cox et al., 2020), les antimicrobiens (Speri et al., 2020) et les probiotiques. La TMF a d'ailleurs suscité beaucoup d'intérêt dans les dernières années, puisque malgré être une approche relativement nouvelle dans la médecine moderne, cette statégie s'est démontré très efficace pour le traitement des ICD récurrentes. La TMF est l'administration de microbiote intestinal isolé d'un donneur sain à un patient infecté. Malgré des résultats prometteurs à moyen et à plus long terme (Barberio et al., 2020), 10 à 15% des patients rechutent suite à l'échec du traitement (Tariq, Saha, Solanky, Pardi, & Khanna, 2020). De plus, la TMF représente un processus long, laborieux et coûteux, ce qui engendre entre autres des délais importants avant de recevoir le traitement (Dubois, Read, O'Brien, & Ling, 2020). Finalement, malgré un processus de dépistage complet ciblant une multitude de pathogènes potentiels, des préoccupations persistent quant à la transmission de maladies au receveur dû au fait que le microbiote transplanté peut autant contenir des bactéries bénéfiques que néfastes qui n'auraient pas été dépistées (Giles, D'Adamo, & Forster, 2019). Ces inquiétudes ont notamment contribué à faire valoir l'utilisation de préparations bactériennes spécifiques et caractérisées en profondeur, comme le sont les probiotiques.

1.6.1 La supplémentation en probiotiques

Les probiotiques sont par définition des "microorganismes vivants qui lorsque consommés en quantité suffisante confèrent un effet bénéfique sur la santé" (Hill et al., 2014). L'utilisation de probiotiques comme stratégie prophylaxique, et dans certains cas, comme traitement des symptômes des ICD ont été explorés. Contrairement à la TMF, l'usage d'une préparation dont la nature est connue et bien caractérisée assure sa sécurité. De plus, les probiotiques représentent une

approche simple, rapide et davantage économique comparativement aux traitements traditionnels (Leal, Heitman, Conly, Henderson, & Manns, 2016). Les allegations santé de probiotiques commerciaux peuvent inclure le support de la santé digestive ou des fonctions intestinales, le maintient d'une flore intestinale normale et saine, la gestion du poids, le traitement et/ou la prévention de la diarrhée, de la constipation, des colites ulcéreuses et des symptômes du SCI (Sniffen, McFarland, Evans, & Goldstein, 2018).

1.6.2 Les mécanismes d'action des probiotiques

Certaines souches spécifiques de probiotiques sont capables de coloniser le TGI et d'exercer des fonctions bénéfiques. Celles-ci incluent la production de substances inhibitrices comme des bactériocines et des AGCCs et l'induction de la relâche de défensines par les cellules de l'épithelium (Flynn et al., 2002; Guinane et al., 2016; Ng, Hart, Kamm, Stagg, & Knight, 2009; Tabasco, Garcia-Cayuela, Pelaez, & Requena, 2009), l'exclusion compétitive via l'inhibition de l'adhésion et de la translocation (Mack, Michail, Wei, McDougall, & Hollingsworth, 1999; Pothoulakis et al., 1993), l'utilisation de nutriments et le métabolisme des acides biliaires, ainsi que la modulation du système immunitaire et de la fonction de barrière de l'épithélium (Parassol *et al*., 2005; Qin *et al*., 2005; Yan *et al*., 2007).

1.6.3 L'utilisation de probiotiques pour le traitement des ICD

L'usage des probiotiques peut être recommandé pour la prévention et/ou le traitement de maladies spécifiques du TGI et de leurs symptômes. Les souches dites classiques et faisant partie des genres *Lactobacillus*, *Bifidobacteria* spp. et *Saccharomyces* ont été parmi les plus étudiées. Comme la perturbation du microbiote et l'abolition de la résistance à la colonisation précède l'ICD, l'usage des probiotiques pour traiter et prévenir les ICD a reçu beaucoup d'intérêt depuis les dernières années de par leur potentiel de soutenir le microbiote intestinal, d'accentuer ses activités anti-pathogènes et ainsi, prévenir la dysbiose et de prévenir l'établissement et/ou la pathogénèse de *C. difficile.*

L'efficacité de nombreuses formulations de probiotiques dans la prévention des ICD a été testée lors d'études cliniques. Ces formulations spécifiques incluent les souches *Saccharomyces boulardii, Lactobacillus* LGG et *Lactobacillus plantarum* 299v et certains mélanges probiotiques comme les combinaisons de *Lactobacillus casei* DN-114 001 (*Lactobacillus casei imunitass*), *Streptococcus thermophilus* et *Lactobacillus bulgaricus* (Actimel, Danone, France),

Lactobacillus acidophilus CL1285, *Lacticaseibacillus casei* LBC80R et *Lacticaseibacillus rhamnosus* CLR2 (Bio-K Plus, a Kerry company, Canada) ainsi que *Lactobacillus acidophilus* NCFM, *Lactobacillus paracasei* Lpc-37, *Bifidobacterium lactis* Bi-07 et *B. lactis* Bl-04 mix (Mills, Rao, & Young, 2018; Na & Kelly, 2011). Malgré que certains souches ou mélanges probiotiques aient démontré leur efficacité dans la réduction de l'incidence des ICD et des symptômes, la crédibilité des études cliniques est parfois questionnée dû entre autres, aux petites tailles des populations à l'étude, aux critères de sélection biaisés, à un double insu insuffisant et à une grande hétérogénécité. Des études plus larges et mieux contrôlées sont alors nécessaires pour confirmer le potentiel des probiotiques dans la prévention des ICD (Mills et al., 2018; Na & Kelly, 2011).

2. OBJECTIFS ET HYPOTHÈSES

Les hypothèses de cette étude étaient que :

- Un microbiote normal et en santé qui inhibe la croissance et la production de toxines de *C. difficile* interfère également avec l'activité du QS de la bactérie, interférence qui serait abolie dans un microbiote en dysbiose.
- Puisque les probiotiques supporteraient le microbiote intestinal en contribuant à sa capacité intrinsèque d'inhibition, la virulence et l'activité du QS de *C. difficile* seraient limitées dans un microbiote en dysbiose ayant reçus des probiotiques.

Les objectifs de cette étude étaient de:

- Développer un modèle *ex vivo* qui permettrait d'étudier une variété d'état du microbiote, soit normal, en dysbiose causée par les antibiotiques et supplémenté en probiotiques.
- Caractériser et comparer l'impact du microbiote sain à celui d'un microbiote en dysbiose et/ou soutenu par les probiotiques sur la modulation du système de QS *agr* de *C. difficile* et sa production de toxines.

Les moyens pour atteindre ces objectifs étaient:

- D'adapter le modèle *in vivo* d'ICD de Chen *et al.* (2008) qui utilise une combinaison d'antibiotiques pour induire la susceptibilité chez la souris et d'y combiner des techniques de co-culture *in vitro* dans le but d'exposer des cellules de *C. difficile* en phase exponentielle au microbiote et d'étudier leur réponse dans le temps. La vialibilité de *C. difficile* a ensuite été mesurée par étalement sur milieux sélectifs et la production de toxines a été mesurée par analyse immuno-enzymatique (ELISA).
- De construire un essai de RT-qPCR grâce à la création et de procéder à la validation de paires d'amorces ciblant les gènes de référence *(gluD, rpoA, rpsJ, adk)*, les gènes de QS *agr* fonctionnels (*agrA2, agrC2, agrB1, agrD1),* ainsi que les deux gènes de toxines (*tcdA, tcdB*) de *C. difficile*. La validation a impliquée l'évaluation de l'efficacité et la spécificité des assays de RT-qPCR ainsi que l'évaluation de la stabilité des gènes de référence.

Les mécanismes derrière l'établissement de *C. difficile* ainsi que sa pathogénèse chez l'hôte ne sont pas encore entièrement illucidés. De plus, la connaissance générale sur le système de QS *agr* et son impact sur la régulation de la virulence de *C. difficile* demeure limitée. Cette étude se voulait de développer un model *ex vivo* dans le but d'exposer des cellules de *C. difficile* a des microbiotes vivants ayant été influencés par des traitements antibiotiques et probiotiques. La combinaison d'un modèle *in vivo* à des techniques de culturomique et d'analyse d'expression génique permettrait de charactériser le role du microbiote intestinal et des probiotiques dans la modulation du système de QS *agr* de *C. difficile.* Autrement, cette investiguation permettrait l'acquisition de nouvelles connaissances sur les mécanismes potentiellement impliqués derrière la résistance à la colonisation du microbiote.

3. MÉTHODOLOGIE

3.1 Conditions de culture

La culture de la souche *Clostridioides difficile* R20291 se faisait dans le milieu de culture TY (*Tryptose Yeast Extract*) et l'étalement se faisait sur les milieux solides *Reinforced Clostridial medium* (RCM) ou *Cycloserine Cefoxitin Fructose Agar* (CCFA) en atmosphère contrôlée composée de 5% H_2, 10% CO_2 et 85% N_2 à 37°C dans un cabinet en anaérobie.

3.2 Modèle animal

Toutes les procédures sur les animaux ont été approuvées par le Comité Institutionel de Protection des Animaux (CIPA) de l'Institut National de la recherche scientifique (INRS, QC, CAN). Des souris femelles C57/BL6 âgées de 7 semaines ont été achetées des Laboratoires Charles River (QC, CAN). Les souris ont été séparées en 4 groupes de traitement soient : aucun traitement (CTRL), supplémentation en probiotiques seulement (PRO), traitement aux antibiotiques seulement (ATB) et combinaison de probiotiques et d'antibiotiques (COMBIN). Le traitement antibiotique des souris tel que décrit dans le modèle d'ICD de Chen *et al.* (2008) a été utilisé. Brièvement, un cocktail composé de kanamycine (0.4 mg/mL), de gentamicine (0.035 mg/mL), de colistine (850 U/mL), de métronidazole (0.215 mg/mL) et de vancomycine (0.045 mg/mL) a été préparé en homogénéisant les antibiotiques dans l'eau autoclavée. Les souris des groupes ATB et COMBIN avaient accès à ce cocktail pendant 3 jours, puis l'eau supplémentée était remplacée par l'eau régulière. Au 5^{e} jour, ces souris ont reçu une injection intrapéritonéale de clindamycine (10 mg/kg). Les souris non traitées aux antibiotiques (groupes CTRL et PRO) ont eu accès à de l'eau régulière sans antibiotique tout au long du traitement et ont reçu une injection de saline (0.9% NaCl). Les souris supplémentées avec des probiotiques (groupes PRO et COMBIN) ont reçu une supplémentation de 1 milliard d'UFC de probiotiques par jour par gavage gastrique pour l'entièreté de l'expérience (5 jours). Les probiotiques ont été préparés en homogénéisant dans du PBS à température ambiante une mixture des souches lyophilisées *L. casei* LBC80R, *L. rhamnosus* CLR2 and *L. acidophilus* CL1285. Les souris non supplémentées en probiotiques (groupes CTRL et ATB) ont reçu un volume équivalent de PBS stérile. Au 6^{e} jour, les souris ont été euthanasiées par asphyxie au CO_2 et dislocation cervicale. Le bloc cecum-colon a rapidement été retiré et placé dans un contenant pour transport de tissus en anaérobie. Les organes ont ensuite été transférés dans le cabinet en anaérobie et les contenus du cecum et du colon ont été isolés et pesés. À ce stade, ces contenus étaient désignés « microbiotes » et ont été utilisés comme tel dans l'étude *ex vivo.*

3.3 Modèle *ex vivo*

Des cellules en phase exponentielle (7 heures de culture) de *C. difficile* R20291 ont été exposées aux différents microbiotes (CTRL, PRO, ATB, COMBIN). Pour ce faire, un volume de 10 mL de culture de *C. difficile* dans le TY en phase mi-exponentielle equivalent à 8.50 ± 0.03 Log_{10} UFC/mL a été ajouté par gramme de microbiote. Une culture de *C. difficile* dans le TY sans présence de microbiote a été utilisée comme culture de référence (REF). Les cultures et co-cultures (REF, CTRL, PRO, ATB et COMBIN) ont ensuite été incubées pendant 5 hrs en atmosphère contrôlée composée de 5% H_2, 10% CO_2 et 85% N_2 à 37°C. Après 1, 3 et 5 hrs, la croissance de *C. difficile* (UFC/mL) et la production des toxines A et B ont été évalués. Le dénombrement des cellules viables de *C. difficile* s'est effectué par une dilution en série dans le PBS et l'étalement sur milieu sélectif CCFA. Les toxines ont été quantifiées dans les échantillons de surnageants filtrés en utilisant un ELISA commercial pour la détection séparée des toxines A et B. Également, pour chaque temps d'exposition, des échantillons de culture ont été prélevés pour l'extraction d'ARN. Ces derniers ont été ajoutés à un volume équivalent d'un mélange d'éthanol 100% et d'acétone (1:1) très froid puis conservés à -80°C jusqu'à l'extraction de l'ARN.

3.4 Méthodes de biologie moléculaire

3.4.1 Extraction et isolement de l'ARN

L'extraction de l'ARN a été réalisée avec le kit RNeasy Mini kit de Qiagen selon les instructions du fabricant avec des modifications mineures, tels que l'utilisation d'un plus grand volume de tampon RLT, l'ajout d'un lavage supplémentaire au RPE et l'utilisation de 3 colonnes par échantillon de 2 mL de culture ou co-culture. L'ADN génomique a été retiré des échantillons d'ARN par un traitement rigoureux du kit Turbo DNA-*free* de Ambion selon les instructions du fabricant. L'intégrité de l'ARN et l'absence d'ADN génomique ont été vérifiées par migration de 250 à 500 ng d'ARN par échantillon et par puit sur gel d'agarose 1%. Les ARN ont au préalable été traités avec le 2X RNA Loading dye (Thermo Scientific) selon les instructions du fabricant.

3.4.2 Amorces

Les amorces utilisées dans cette étude ont été créées via l'utilisation des logiciels en ligne Primer3 (Untergasser and Nijveen, 2007) et OligoQuest (IDT). Un total de 3 à 4 paires d'amorces ont été développées à des fins de validation pour les gènes d'intérêt *agrA, agrC, agrB, agrD, tcdA* et *tcdB* et les gènes de référence (GRs) *gluD, rpoA, rpsJ* et *adk.* Les amorces sélectionnées pour cette étude sont présentées dans le Tableau 1 (*Table* 1). Une série d'étapes de validation a été effectuée afin de sélectionner les paires d'amorces optimales, débutant par gradient de température. Par la suite, un test d'efficacité a été réalisé en produisant une courbe standard en 8 points d'un ADN de référence de *C. difficile* dilué 2 ou 5 fois en série. La spécificité des amplifications a été validée par l'analyse des courbes de dissociation ou « *melting curves* » et en validant la taille attendue des fragments amplifiés par migration sur gel d'agarose 2% de 1 µL de produits PCR. Finalement, la stabilité des GRs a été vérifiée en utilisant l'outil de sélection des gènes de référence du logiciel CFX Maestro (Bio-Rad). Pour ce faire, des échantillons d'ADN complémentaire (ADNc) de *C. difficile* en culture pure et en co-culture à différents temps de culture ont été utilisés pour représenter un lot de conditions différentes. La sélection des GRs les plus stables a été basée sur les recommandations du logiciel.

3.4.3 Réaction de RT-qPCR

L'ARN prrifié a été convertie en ADNc par réaction de transcription inverse (RT) en utilisant la trousse QuantiTect Reverse Transcription de Qiagen selon les instructions du fabricant. Un total de 1000 ng d'ARN a été utilisé dans chaque réaction de RT. Pour le contrôle négatif de RT (NRT), l'enzyme transcriptase a été substituée par de l'eau sans nucléase. Un total de 40 ng d'ADNc a été utilisé par réaction de qPCR dans un volume réactionnel de 10 µL en utilisant le 2x SsoAdvanced Universal SYBR Green Supermix de Bio-Rad et 0.2 ou 0.3 ρmol d'amorces sens et anti-sens. Des plaques de 96 puits ont été chargées avec les échantillons inconnus en triplicat technique et chargées avec les contrôles NRT et sans ADNc « *no template* » (NTC) en duplicata technique. Un étalonnage « *inter-run »* (IRC) a été utilisé en triplicata dans toutes les plaques.

3.5 Analyse des données et analyse statistique

L'expression normalisée relative a été calculée en utilisant la méthode $2^{-\Delta\Delta Ct}$. L'expression des gènes d'intérêt a été normalisé sur l'expression des GRs *rpoA* et *adk*. Les données d'expression génique sont présentées comme le changement ou « *fold-change »* d'expression, relatif à l'expression mesurée dans la culture de référence ou le groupe CTRL selon le cas. Les résultats

présentés sont des moyennes de réplicats biologiques de trois expériences indépendantes (n=3). Les données ont été analysées en utilisant le logiciel Prism 9 (GraphPad). Les analyses statistiques Student T-test et *one-way* ANOVA avec posthoc test Tukey multiple comparaison ont été réalisées pour la comparaison des changements d'expression entre deux groupes ou entre plus que deux groupes respectivement. Les moyennes des changements d'expression entre les groupes ont été comparées entre elles pour chaque temps d'exposition. Le seuil alpha inférieur ou égal à 0.5 a été sélectionné pour les analyses statistiques.

4. RÉSULTATS ET DISCUSSION

4.1 Développement d'un essai de qPCR pour l'analyse d'expression génique du système de QS *agr* chez *C. difficile*

4.1.1 Spécificité

Due à la grande complexité des échantillons d'ARN des co-cultures de *C. difficile* avec le microbiote intestinal, les amorces devaient démontrer une très grande spécificité pour les gènes de *C. difficile* afin d'éviter des amplifications non spécifiques. Dans le cadre de cette étude, les amorces étaient dites spécifiques lorsque l'intervalle des Cq entre l'amplification dans le contrôle positif et l'amplification dans le contrôle négatif était supérieur à 4 ($\Delta Cq < 4$, où $\Delta Cq = Cq_{neg} - Cq_{pos}$). Un ΔCq inférieur ou égal à 4 engendrait l'exclusion de la paire d'amorces. Les paires d'amorces sélectionnées pour cette étude sont présentées dans le Tableau 2. Les paires sélectionnées ont démontré des ΔCq supérieurs à 7.01. Les amplifications du contrôle positif ont résultés en des valeurs de Cq variant entre 18.37 et 27.90. Toutes les amplifications du contrôle négatif ont résultés en une valeur de Cq supérieure à 31.70. À la lumière de ces résultats, ces paires d'amorces ont été considérées comme étant spécifiques pour l'organisme d'intérêt, soit *C. difficile.*

4.1.2 Efficacité

Suite au test de spécificité, un test d'efficacité a été réalisé afin d'assurer que l'essai réponde aux exigences de performance analytique. Ainsi, les critères d'évaluation incluaient une efficacité (E) entre 90% et 110%, une pente entre -3.6 et -3.1 et un $R^2 \geq 0.995$. Selon les paires d'amorces, des courbes standard ont été produites en faisant des dilutions en série 1:2 ou 1:5 d'un ADN de référence de *C. difficile.* Les données d'efficacité des paires d'amorces sélectionnées suite au test de spécificité peuvent être visualisées dans le Tableau 3. Les résultats démontrent que toutes les paires d'amorces rencontrent les exigences. À noter que dû à des résultats d'efficacité insatifaisants pour la paire agrC2_MB3 à une concentration d'amorce initiale de 0.3 ρmol (E = 132.8%, résultat non montré), le test a été refait avec une nouvelle concentration de 0.2 ρmol, ce qui a mené à une performance adéquate (E = 101.1%, Table 3) et le respect des exigences. Ce test d'efficacité a démontré que les réactions PCR sont performantes. À noter également, que pour chaque paire d'amorces, la portion linéaire de la réaction (*dynamic linear range*) couvrait au minimum 6 ordres de magnitude (résultats non montrés), ce qui respecte les recommandations du MIQE, qui stipule qu'au moins 3 ordres de magnitudes devraient être couverts par la portion linéaire (Bustin et al., 2009).

4.1.3 Stabilité des gènes de référence

La stabilité des gènes de référence a été testée afin d'établir s'ils étaient adéquats pour normaliser les expressions des gènes d'intérêt. Ce test a été effectué en préparant un lot d'ARN de référence représentant une variété de conditions, soient différents types de culture (*i.e.* culture pure de *C. difficile* ou co-culture *C. difficile* et microbiote) et différents temps de culture (*i.e.* 2 et 6 hrs). Les résultats d'amplification ont été analysés avec l'outil de sélection des GRs du logiciel CFX Maestro. La Table 4 démontre la stabilité des quatre GRs amplifiés avec les paires d'amorces démontrant les meilleurs résultats de spécificité et d'efficacité. Les résultats démontrent que les gènes *rpoA, rpsJ* et *adk* sont exprimés de façon stable à travers les conditions incluses dans le test. À l'inverse, le gène *gluD* a été trouvé instable dans ces conditions. Suivant les recommandations du logiciel, les gènes *rpoA, rpsJ* et *adk* ont été considérés comme GRs idéaux. Comme la normalisation sur un seul GR est considérée comme inacceptable selon les recommandations du MIQE et que la normalisation sur plus d'un GRs est fortement recommandée (Bustin et al., 2009), les trois GRs idéaux ont été utilisés dans les expériences suivantes.

4.1.4 Validation

Après chaque étape d'optimisation, les paires d'amorces étaient validées par migration sur gel d'agarose 2% des produits d'amplification afin de s'assurer que les amplifications produisaient les produits de tailles attendues. Les Figures 5-7 illustrent des résultats de migration représentatifs pour chaque paire d'amorces, et démontrent que les produits PCR ont les tailles attendues et qu'un seul produit est amplifié. Également, l'analyse des courbes de dissociation (*melting curve*) a permis de confirmer la présence d'un seul pic pour chaque paire d'amorce et de confirmer l'absence de la formation de dimères d'amorces qui pourraient nuire à l'efficacité de la réaction (Figures 8-10).

4.2 Modulation du système de QS *agr* de *C. difficile* suite à l'exposition de microbiote intestinal

4.2.1 Croissance de *C. difficile* en présence de microbiote intestinal

La viabilité de *C. difficile* a été affectée légèrement par la présence des microbiotes (Figure 12). Après 1 h d'exposition, le taux de croissance de *C. difficile* était similaire dans les 4 groupes; CTRL, PRO, ATB et COMBIN et dans la culture de référence (REF) avec une augmentation moyenne de 0.19 ± 0.02 Log_{10}UFC/mL après 1 hr. Cependant, à partir de 3 hrs on note que la présence de microbiote a négativement impacté l'expansion de *C. difficile.* Au bout des 5 hrs d'exposition, on observe une différence significative des niveaux de croissance entre la culture de référence et les groupes CTRL et ATB ($P \leq 0.01$) et PRO et COMBIN ($P \leq 0.05$). Au final, le nombre moyen de cellules viables de *C. difficile* en présence des microbiotes était inférieur de 0.17 ± 0.08 Log_{10}UFC/mL comparativement à la culture de référence. En général, les microbiotes sains (CTRL et PRO) n'ont pas été davantage inhibiteurs que les microbiotes en dysbiose supplémentés en probiotiques ou non (COMBIN et ATB; $P > 0.05$). Également, le pH n'a pas varié de façon significative durant l'expérience et n'était pas différent entre les groupes ($P = 0.47$; Table 5). Une raison pouvant expliquer la faible inhibition de croissance de *C. difficile* en présence de microbiote serait que les bactéries provenant des microbiotes consomment également les nutriments du milieu, et peut être même de manière plus efficace, ce qui réduit la disponibilité des nutriments pour *C. difficile.* Sinon, malgré que ces différences soient statistiquement significatives, on ne considère pas les variations mesurées (<1 Log) comme étant significatives d'un point de vue biologique (Prof. M. Lacroix, communication personnelle).

Aucune différence n'a pu être détectée entre les expositions à des microbiotes sains comparativement à des microbiotes en dysbiose, ce qui est en contradiction avec les études précédentes dans lesquelles une inhibition de croissance de *C. difficile* était mesurée en présence de microbiote sain et non en présence de microbiote traité aux antibiotiques (Borriello & Barclay, 1986; Borriello et al., 1988; Nerandzic & Donskey, 2011). Différentes raisons peuvent expliquer cette disparité entre les tendances observées dans cette présente étude et ailleurs. Dans le cas de cette étude, certaines conditions de culture sont différentes notamment quant à l'utilisation du milieu de culture TY (*vs* le PBS), un large inoculum au moment de l'exposition au microbiote (8.50 Log_{10}UFC/mL) et une courte période d'expérimentation (5 hrs *vs* 24-48 hrs). Ainsi, la présence de nutriments apportés par le TY a très probablement eu un effet, autant sur *C. difficile* que sur les bactéries du microbiote, ce qui pourrait avoir atténué l'impact spécifique de chacun des microbiotes. Également, on ne peut nier le fait que des tendances différentes auraient pu être

observées sur une période de temps plus étendue, au-delà de 5 hrs d'incubation. Cependant, comme l'intérêt de cette étude était principalement axé sur la modulation du QS, dont l'activité maximale serait en fin de phase exponentielle, l'étude d'une plus longue exposition au microbiote allait au-delà de la portée de ce projet.

4.2.2 Production des toxines et expression de *tcdA* et *tcdB* en présence de microbiote intestinal

Les niveaux de toxine B n'ont pas atteint les seuils de détection du kit et n'ont donc pas pu être évalués dans cette étude. En ce qui concerne la toxine A, les microbiotes sains (CTRL et PRO) n'ont pas inhibé la production de toxine A et ont permis des productions légèrement supérieures (CTRL : 4.48 ng/mL, SEM 1.55 et PRO : 4.47 ng/mL, SEM 0.73) comparativement aux niveaux mesurés dans la culture de référence (3.45 ng/mL, SEM 1.47) et dans les groupes ATB et COMBIN (ATB : 3.15 ng/mL, SEM 0.76 et COMBIN : 3.64 ng/mL, SEM 0.98) (Figure 13). Bien que les différences dans les niveaux de production de toxine A n'étaient pas significatives ($P > 0.05$), ces résultats sont appuyés par l'analyse comparative de l'expression des gènes *tcdA* et *tcdB.*

En effet, comparé à la culture de référence, l'expression de *tcdA* et *tcdB* était d'environ 2 fois supérieure dans le groupe CTRL après 1 h, ce qui aurait pu contribuer à une plus grande production de toxine dans ce groupe au bout de 5 hrs (Figure 17). Autrement, comparé au groupe CTRL, il a été observé que l'expression relative de *tcdA* et de *tcdB* était diminuée après 1h dans les groupes ATB (*tcdA:* -5.56 fois, $P = 0.07$, *tcdB:* -3.50 fois, $P = 0.13$) et COMBIN (*tcdA:* -12.5 fois, $P \leq 0.05$, *tcdB:* -6.67 fois, $P = 0.06$) respectivement, tandis qu'elle était similaire dans le groupe PRO (Figure 22). Ces résultats seraient toujours en contradiction avec des études précédentes qui avaient observé une diminution de la production de toxine en présence de microbiote sain et une augmentation en présence de microbiote en dysbiose (Borriello & Barclay, 1986; Borriello et al., 1988; Nerandzic & Donskey, 2011). Au final, les microbiotes sains n'ont pas inhibé la production de toxine par *C. difficile* comme il était attendu. De plus, des niveaux légèrement inférieurs de toxine ont été mesurés en présence de microbiote en dysbiose.

Une explication plausible à ces résultats serait que l'environnement nutritionnel, qui aurait été modifié par le microbiote, pourrait être davantage impliqué dans les tendances observées comparativement à des mécanismes directs du microbiote envers *C. difficile*. L'implication de la disponibilité en nutriments de l'environnement et du métabolisme a d'ailleurs été démontrée chez *C. difficile* comme ayant un impact sur la virulence. Des régulateurs de transcription comme CodY et CcpA seraient impliqués dans l'inhibition des toxines via la répression du régulateur positif *tcdR*

dans les environnements plus riches en nutriments comme des acides aminés à chaîne ramifiée (BCAA), GTP et glucose (Ana Antunes et al., 2012; Antunes, Martin-Verstraete, & Dupuy, 2011; Dineen, McBride, & Sonenshein, 2010; Dupuy & Sonenshein, 1998; Nawrocki, Edwards, Daou, Bouillaut, & McBride, 2016). On pourrait alors suggérer que la présence d'un microbiote sain, contenant une population bactérienne très diversifiée et des membres clés quant à l'utilisation de certains nutriments, ai contribué à diminuer la disponibilité des nutriments dans les co-cultures CTRL et PRO. Ceci aurait alors engendré une plus grande dé-répression de *tcdR,* ce qui aurait activé la production de toxines de manière plus importante que dans le cas d'un microbiote en dysbiose (Figure 23).

4.2.3 Analyse comparative des gènes de QS *agr* en présence de microbiote intestinal

Les résultats démontrent que tous les gènes *agr* étaient sur-exprimés après 3 et 5 hrs d'exposition d'un microbiote sain (CTRL) par rapport aux conditions standards de culture (REF) (Figure 16). Toutefois, aucune de ces sur-expressions n'a été considérée comme statistiquement significative ($P > 0.05$). De manière surprenante, les sur-expressions mesurées quant à *agrB* et *agrD* (changement d'expression variant entre +6.62 et +16.14 fois) qui étaient généralement de plus grande amplitude que les expressions mesurées pour *agrAC* (changement d'expressions variants entre +4.82 et +8.18 fois), n'ont tout de même pas démontré de différence significative comparativement à la culture de référence. Malgré cela, ces résultats démontrent que *C. difficile* répond bel et bien à son environnement et que son système de QS *agr* semble être modulé positivement en présence de microbiote.

En comparant les microbiotes entre eux, les résultats démontrent que les gènes *agrA* et *agrC* ont été régulés positivement et négativement dans les groupes ATB et COMBIN respectivement après 3 hrs (+/-2 fois, $P > 0.05$; Figures 18-19). Le gène *agrB* a été fortement et significativement sur-exprimé après 3 hrs (+5.11 fois, $P \leq 0.05$) et 5 hrs (+6.37 fois, $P \leq 0.01$) dans le groupe ATB (Figure 20). De manière très similaire, *agrD* a été également sur-exprimé de façon significative dans ce groupe après 3 hrs (+4.74, $P \leq 0.05$) et après 5 hrs (+7.84 fois, $P \leq 0.01$) (Figure 21). De manière intéressante, *agrB* et *agrD* n'ont pas été régulés différement dans le groupe COMBIN relativement au groupe CTRL après 3 hrs. Après 5 hrs, ces gènes étaient cependant sur-exprimés dans ce groupe (Figures 20-21).

Après 3 hrs, mis à part pour *agrC* ($P = 0.11$), l'expression relative des gènes de QS était significativement inférieure dans le groupe COMBIN comparé au groupe ATB ($P \leq 0.05$). Toutefois, ces différences significatives n'ont pas été observées de nouveau après 5 hrs.

Contrairement à *agrAC* dont les expressions relatives sont demeurées similaires dans tous les groupes au fil du temps, les sur-expressions de *agrBD* étaient de plus en plus prononcées dans le temps dans les groupes ATB et COMBIN. Ces résultats suggèrent que la régulation positive des gènes *agrBD* serait corrélée positivement avec la durée d'exposition.

De manière générale, il a été observé que l'ensemble des gènes de QS ont été sur-exprimés lorsque *C. difficile* était exposé au microbiote en dysbiose (ATB), suggérant que ce système à été davantage stimulé dans cet environnement comparativement à un microbiote sain (CTRL). Chez *S. aureus,* il a été démontré que le système *agr* pourrait être inhibé dans des environnements riches en nutriments via une répression indirecte par le régulateur transcriptionnel CodY, suggérant que l'activation d'*agr* est étroitement contrôlée en réponse à des signaux environnementaux (Roux, Todd, Velazquez, Cech, & Sonenshein, 2014). Plusieurs autres régulateurs d'*agr* ont d'ailleurs été identifiés chez *S. aureus* (Kavanaugh & Horswill, 2016). Or, dans cette présente étude, il semblerait que le système *agr* soit stimulé plutôt qu'inhibé en présence d'un environnement que l'on suppose être plus riche en nutriments (ATB), ce qui contredirait ce qui a été découvert chez *S. aureus*.

4.2.4 Impact de la supplémentation en probiotique

Finalement, la présence de probiotiques semble inhiber la régulation positive des gènes *agrB* et *agrD* (Figures 20-21), ainsi que les gènes *tcdA* et *tcdB* (Figure 22) tel qu'observé dans un microbiote en dysbiose, suggérant un certain rôle d'interférence des probiotiques, direct ou indirect, sur le système *agr* et dans la virulence de *C. difficile.* Toutefois, cette étude n'a pas permis d'établir si l'effet observé découle de la modulation des probiotiques sur l'environnement et/ou sur le microbiote intestinal ou s'il est attribuable à un effet direct et antagoniste des probiotiques contre *C. difficile* dans les co-cultures. Autrement, la présence de probiotiques dans un microbiote normal (PRO) n'a pas eu d'effet significatif sur la croissance ou la viabilité de *C. difficile* (Figure 12), l'expression de *tcdA* ou *tcdB* (Figure 22) et la production de toxine par *C. difficile* (Figure 13). Ceci pourrait suggérer que l'impact des probiotiques sur le microbiote ne serait pas suffisamment important dans le contexte où le microbiote est déjà sain, riche et diversifé. Cependant, bien que les différences n'étaient pas significatives ($P > 0.05$), l'expression de *agrA* et *agrC* est bel et bien demeurée inhibée en tout temps dans le groupe PRO relativement au groupe CTRL. À notre connaissance, une inhibition du système *agr* dans le contexte d'une interférence du QS, n'a pas été identifé pour le moment chez *C. difficile.* Les résultats semblent toutefois pointer vers une régulation négative du locus *agr2* en présence de probiotiques et/ou de produits dérivés des probiotiques.

5. CONCLUSION ET PERSPECTIVES

Les connaissances actuelles concernant l'impact de l'environnement et du microbiote intestinal sur le système de QS des pathogènes, comme *C. difficile,* ainsi que l'impact subséquent sur leur virulence sont limitées. Cette étude se voulait d'investiguer l'effet de microbiotes normal et sain, en dysbiose suite au traitement antibiotiques et supplémenté en probiotiques, sur le système de QS *agr* de *C. difficile* et sa virulence. Notamment, ceci permettrait de mieux comprendre les processus et les régulations contribuant à l'établissement de *C. difficile* et l'expression de ses facteurs de virulence suite à la dysbiose intestinale.

Les différents microbiotes ont permis d'étudier les réponses de *C. difficile* dans des environnements davantage représentatifs de son réservoir naturel d'infection : le tractus gastro-intestinal, permissif ou non à l'infection. Effectivement, grâce au développement d'une plateforme de RT-qPCR pour l'analyse d'expression génique du système de QS *agr* chez *C. difficile* permettant d'obtenir des amplifications efficaces et spécifiques des gènes de la cible d'intérêt (*i.e., C. difficile*), les résultats ont pu démontrer que l'activité d'*agr* est régulée positivement en présence de microbiote, ce qui suggère que *C. difficile* répond bel et bien à son environnement typique d'infection. Également, il a été démontré que les gènes impliqués dans la production du signal, *agrB* et *agrD,* sont fortement surexprimés en présence de microbiote en dysbiose, ce qui supporterait l'idée qu'il existe des rôles distincts potentiels pour ces deux protéines allant au-delà de la production de signal dans un contexte de *quorum sensing* tel que suggéré ailleurs (Ahmed et al., 2020). Également, étant donné que les expressions d'*agrBD* (du locus *agr1*) et *agrAC* (du locus *agr2)* suivaient des tendances de régulation distinctes, ceci pourrait suggèrer que les loci *agr1* et *agr2* ne sont pas co-régulés et seraient potentiellement régulés en réponses à des stimulis distincts. En général, les résultats de cette étude permettraient de soutenir l'idée que le microbiote aurait une influence sur l'environnement nutritif du tractus gastro-intestinal et que la régulation de *agr* et des toxines chez *C. difficile* seraient influencée par l'environnement nutritif dans lequel il se retrouve. Par contre, cette étude n'a pas pu démontrer une association entre la surexpression des gènes de QS et une hausse de la production de toxine A, ce qui suggère que des facteurs additionnels sont impliqués dans cette régulation. Finalement, les différences significatives entre les expressions relatives des gènes *agrBD* entre les groupes ATB et COMBIN suggèrent une forme potentielle d'interférence du système de QS qui serait associée à la présence d'une formulation probiotique spécifique dans le microbiote COMBIN.

Comme ce travail aura servi à étudier la faisabilité de ce modèle, plusieurs informations utiles notamment sur la variabilité biologique des échantillons ont pu être obtenues. Également,

certains obstacles et limitations ont été soulevés et pourront être addressés dans les expériences futures. Parmi ceux-ci, se retrouve le fait que peu de réplicats ont été incluent dans l'étude, soit 3 réplicats par groupe. Comme cette étude a démontrée qu'il existe une grande variabilité biologique au sein des groupes, une analyse de puissance statistique pourrait être exécutée afin d'estimer la taille d'échantillon appropriée pour détecter l'effet des différents traitements sur les variables étudiées. Autrement, ni la viabilité du microbiote intestinal murin ni celle des souches probiotiques n'a été contrôlé dans cette expérience. La quantification des bactéries du microbiote et des probiotiques aurait pu être réalisée avec l'étalement des cultures sur un éventail de milieux solides sélectifs ciblant certains groupes bactériens spécifiques comme les Bifidobactéries et les bactéries produisant l'acide lactique (LAB), une technique décrite par de Carvalho *et al.* (2021). En outre, les ARN extraits représentaient des niveaux variables de dégradation (voir Figures 14-15). Il n'a pas été possible de refaire des extractions d'ARN subséquentes dû à des volumes limités d'échantillon. Compte tenu du biais potentiellement causé par des ARN de mauvaise qualité, une attention particulière a été portée quant à l'analyse des courbes d'amplification pour s'assurer de la reproductibilité entre les réplicats. Cependant, il est indéniable que l'utilisation d'ARN intact et de très bonne qualité est souhaitable pour obtenir des résultats d'expression robustes et fiables. Ainsi, comme la qualité des ARNs utilisés dans cette expérience ne soit pas conforme avec les recommendations du MIQE, du travail supplémentaire dans l'optimisation de la technique d'extraction d'ARN est alors nécessaire. Entre autres, des stratégies telles que l'utilisation de solutions comme le RNAlater (Invitrogen) ou le RNAprotect (Qiagen) et l'utilisation d'inhibiteurs des RNases pourraient être explorées afin de protéger plus efficacement l'ARN de la dégradation.

Finalement, les expériences futures pourraient se concentrer sur l'étude de l'expression de régulateurs transcriptomiques impliqués dans la réponse aux nutriments, tel que CodY et CcpA, ainsi que d'investiguer et de comparer les concentrations en nutriments telles que les sources de carbones, acides aminés à chaîne ramifiée, les AGCCs et autres métabolites dans les différents groupes. Il sera aussi intéressant de mieux comprendre comment le probiotique module le microbiome et le métabolome intestinaux afin de mieux expliquer son rôle en présence ou en absence du traitement antibiotique. Connaître l'impact des différents traitements sur la modulation des populations bactériennes du microbiote des souris ainsi que la fluctuation de ces populations en co-culture avec *C. difficile* via l'analyse des populations (gène codant pour l'ARNr 16S) permettra de mieux comprendre comment les environnements intestinaux ont été influencés. Finalement, malgré que l'implication de ce système dans la virulence de *C. difficile* soit controversée, il serait très intéressant d'étudier le second système de QS, soit le système LuxS/AI-2. En effet, même dans le cas de figure où le AI-2 produit par *C. difficile* ne serait qu'un produit

secondaire du métabolisme de la méthionine, il serait probable que *C. difficile* puisse sentir l'environnement qu'il partage avec d'autres membres bactériens et qu'il soit apte à adapter ses comportements en conséquence (Pereira, Thompson, & Xavier, 2013).